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Characterization of human memory NK cells *ex vivo* and study of therapeutic monoclonal antibody capability to promote their expansion *in vitro*

PhD School in Immunological, Haematological and Rheumatological Sciences
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INTRODUCTION

1. HUMAN NATURAL KILLER CELLS

Natural Killer (NK) cells are lymphocytes belonging to the innate branch of the immune system. They are included in the innate lymphoid cell (ILC) hemopoietic lineage, a recently identified heterogeneous group of lymphoid effector cells, largely tissue-resident and important orchestrators of early immune responses (Mjösberg J and Spits H, 2016; Vivier E et al., 2008).

NK cells have been initially identified in the peripheral blood (PB) by the unique ability to kill tumor cells without major histocompatibility complex (MHC) restriction and any prior activation, thus defined “natural” (Jondal M and Pross H, 1975; Kiessling R et al., 1975; Pross HF and Jondal M, 1975). In the last 40 years, our perception of NK cells is dramatically changing as, beside their ability to kill transformed cells, they have been recognized to kill virus-infected, damaged and allogenic cells, and also to contribute to immunoregulation and participate in tissue remodelling (Cooper MA et al., 2001a; Vivier E, 2006). Currently, over 30,000 NK cells subsets displaying peculiar features can be identified, reflecting the huge plasticity and diversification of this population (Horowitz A et al., 2013). Moreover, in the last decade, the characteristic of immunological memory has been recognized to the NK cell population, challenging the rigid classification that relegates them in the innate immune system (Rölle A and Brodin P, 2016).

1.1 Main biological and functional characteristics

NK cells represent 5-15% of circulating lymphocytes in humans, although significant populations permanently reside in tissues such as bone marrow, liver, spleen, gut, skin, lungs, and uterus during pregnancy. Moreover, NK cells were also found in other tissues: kidney, joints, and breast, under pathological conditions (Law BMP et al., 2017; Mamessier E et al., 2011; Teo T et al., 2015). Their different localization reflects their phenotypic and functional heterogeneity (Björkström NK et al., 2016; Peng H and Tian Z, 2017; Vivier E, 2006).

In the lungs, for example, NK cell ability to respond rapidly to infection suggests an important role for these cells in airway protection. However, it is emerging that they

are also important in the regulation of chronic infections, as tuberculosis, and chronic inflammatory disorders, as asthma, and lung fibrosis (Culley FJ, 2009; Marquardt N et al., 2017). The liver is a tolerogenic environment, where NK cells are important with their anti-viral properties, for example in HBV and in HCV infection (Aw Yeang HX et al., 2017; Maini MK and Peppa D, 2013; Peng H and Tian Z, 2017).

During pregnancy, uterine NK cells importantly contribute to spiral artery remodelling and trophoblast invasion, by producing cytokines, and angiogenic and growth factors (Faas MM and de Vos P, 2017; Gamliel M et al., 2018; Moffett-King A, 2002).

NK cells are an important component of tumor immunosurveillance; they can be recruited in the tumor microenvironment where they contribute to malignant cell killing and also exert immunoregulatory function (Larsen SK et al., 2014; Malmberg KJ et al., 2017; Stojanovic A and Cerwenka A, 2011).

In animal models, they have been recognized as important in controlling tumor growth and metastasis spreading (Guillerey C and Smyth MJ, 2015; Hayakawa Y and Smyth MJ, 2006; Kärre K et al., 1986; Ljunggren HG and Kärre K, 1985). Indeed, deficiencies in NK number (Kim S et al., 2000) and function (Talmadge JE et al., 1980), or NK cell depletion (Seaman WE et al., 1987), increase the rate of tumor and metastasis.

Retrospectives studies correlate a lower NK cell activity with tumor incidence (Imai K et al., 2000). While, NK cell infiltrate in gastrointestinal sarcoma (Delahaye NF et al., 2011) and in lung metastasis correlates with a better prognosis (Remark R et al., 2013). Moreover, the defective expression of NK activating receptors has been correlated with the incidence of multiple myeloma (Fauriat C et al., 2006), hepatocellular carcinoma (Jinushi M et al., 2005), metastatic melanoma (Konjević G et al., 2007) and other tumors. All these reports suggest a direct correlation between NK cells presence/functionality with tumor incidence and outcome (Guillerey C and Smyth MJ, 2015).

NK cell most recognized functional abilities are target cell killing and the secretion of a vast array of cytokines and chemokines. NK cytotoxic activity can be exerted through three main lytic mechanisms, which are shared with cytotoxic T lymphocytes (CTL):

1) The exocytosis, into a tight cytotoxic synapse, of granules containing perforin membrane-disrupting molecule and granzyme serine-proteases, resulting in the apoptosis of the target cell, by caspase cascade activation. Moreover, granzyme-induced target cell death can be also induced by a caspase-independent pathway that leads to mitochondria swelling and disruption (Arias M et al., 2017; Chowdhury D and Lieberman J, 2008; Estaquier J et al., 2012).
2) Receptor-dependent target cell apoptosis, mediated by TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL or CD178), which are induced on the surface of activated NK cells. These death ligands bind to their respective death receptors, Fas (CD95) for FasL and TRAIL-R1/2 for TRAIL, on target cell surface, promote receptor oligomerization and the death domain (DD)-mediated recruitment of Fas-Associated Death Domain (FADD) adaptor protein, which in turn activates the caspase apoptotic pathway (Martínez-Lostao L et al., 2015; Peter ME and Krammer PH, 2003).
3) Tumor necrosis factor (TNF)-α is secreted in large amount by NK cells. It is produced as a membrane protein that is later released in a soluble form by the action of TACE metalloprotease. TNF-α is recognized by two receptors: TNFR1, possessing conserved death domains (DD) and expressed at low level in all cell types, and TNFR2, regulated and expressed only in specific cells, as neurons, endothelial and immune cells. Upon ligand binding by TNFR1, the activation of adapter proteins through intracellular DD converges on FADD activation, as for TRAIL and FasL, and consequently in the formation of IIa and IIb complexes that induce caspase activation and subsequent apoptosis (Aggarwal BB et al., 2012; Kalliolias GD and Ivashkiv LB, 2016). Moreover, TNFR1 downstream signalling pathways can also promote the formation of IIc complex, which leads to necroptosis (Silke J et al., 2015).

Upon activation, NK cells are also able to secrete a vast array of cytokines and chemokines which exert a strong immunomodulatory activity, and orchestrate traffic and functional responses of other immune components in the inflammatory

sites (**Figure 1**): interferon γ (IFN-γ), TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and the chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, the factor regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), among others (Fauriat C et al., 2010a).

IFN-γ, or type II interferon, plays critical roles in both host defence and immune regulation, by exerting multiple and pleiotropic effects (Castro F et al., 2018; Schoenborn JR and Wilson CB, 2007; Schroder K et al., 2004).

IFN-γ receptor is expressed in nearly all cell types and composed by two subunits, IFNGR1 and IFNGR2 that, upon ligand binding, rapidly activate JAK1 and JAK2 Janus tyrosine kinases, responsible for the activation of STAT-1, a member of the STAT (signal transducer and activator of transcription) protein family; STAT-1 then translocates into the nucleus, and promotes the transcription of IFN-γ-inducible genes. Most of them are transcription factors themselves, such as interferon regulatory factor (IRF)-1 -2 and -9, which promote, in turn, the transcription of downstream genes. In parallel, IFN-γ induces a JAK/STAT-independent pathway, involving MAP kinases. As a result, global changes are induced in the cell, influencing cell cycle, adhesion and functions (Castro F et al., 2018; Schroder K et al., 2004). This cytokine is also endowed with anti-tumor activity, by inhibiting cell proliferation, via upregulation of p21 and p27, or through apoptosis induction (Mojic M et al., 2017). For instance, IFN-γ induces IRF1, a tumor suppressor gene, which in turn reduces B-cell lymphoma-2 (Bcl-2) and increases Bcl-2 homologous antagonist/killer (Bak). As a result of cytochrome C release from mitochondria and reactive oxygen (ROS) and nitrogen species production, apoptosis is induced (Aqbi HF et al., 2018).

Moreover, IFN-γ influences antigen processing and presentation by both MHC class I and class II molecules, by inducing the replacement of some of the proteasome subunits; by promoting the synthesis of transporter associated with antigen processing (TAP) 1 and 2, which deliver cytosolic peptides in the endoplasmic reticulum for their processing; by up-regulating MHC subunits (Castro F et al., 2018; Schroder K et al., 2004).

IFN-γ acts on many immune cell populations, mainly by: a) resulting in an increased microbe ingestion and destruction by phagocytes, either by directly targeting macrophages, and indirectly by up-regulating CD40 ligand on T_H1 cells;

b) polarizing the immune response toward a T_H1 response, by promoting $CD4^+$ T cells differentiation in T_H1 subset, and, in parallel, by blocking T_H2 and T_H17 subset differentiation; c) promoting the switch to opsonizing IgG subclasses by activated B cells (Schroder K et al., 2004); d) enhancing NK and CTL cytotoxicity.
However, several studies have outlined the role of chronic IFN-γ production in tumorigenesis and in tumor immune evasion. Under homeostatic conditions, IFN-γ up-regulates genes designed to limit tissue damage and facilitate tissue repair upon resolution of inflammation. Chronic exposure to IFN-γ induces an epigenetic signature leading to the expression, in the tumor microenvironment, of immune suppressive molecules, such as programmed death-1 (PD-1) and its ligands PD-L1 and PD-L2, cytotoxic T-Lymphocyte antigen-4 (CTLA-4), that induce a hypofunctional/exhausted phenotype in T lymphocytes and NK cells (Benci JL et al., 2016). IFN-γ also up-regulates the expression of HLA-E non canonical HLA molecule that, by interacting with inhibitory receptors, promotes tumor escape from CTL and NK cells (Derré L et al., 2006).
Besides the previously described direct cytotoxic effects on target cells through the IIa, IIa and IIc complexes downstream of TNFR1 receptor, **TNF-α** also exerts immunoregulatory activity. Through TNFR1-induced complex I assembly, comprising adapter molecules TRADD and TNFR-associated factor 2 (TRAF2), signals converge on mitogen-activated kinase (MAPK) cascades, and Activator protein 1 (AP1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, important promoters of inflammation, host defence, cell proliferation and survival (Aggarwal BB et al., 2012; Brenner D et al., 2015). TNFR2, instead, is believed to bind preferentially to the transmembrane form of TNF-α. This receptor lacks DD and it is not able to induce cell death, instead it promotes local homeostatic effects, such as cell survival and tissue regeneration, by recruiting TRAF2 and the formation of complex I (Kalliolias GD and Ivashkiv LB, 2016).
GM-CSF is an important hematopoietic growth factor and modulator, mostly involved in inducible hemopoiesis. It mainly acts on multipotent progenitor cells to drive the production of granulocytes and monocytes. It also induces the recruitment of monocytes, induces their differentiation to macrophages and dendritic cells (DCs), depending on other cytokines present in the

microenvironment (Becher B et al., 2016).

NK cells can be triggered to perform their effector functions either upon cell-cell contact, mediated by a vast array of surface receptors (vide infra), and by the action of several cytokines, which tune their activation status together with other aspects of their biology, as development, homeostasis and proliferation. The main cytokines whose receptors are expressed on human NK cells are: IL-2 family (IL-2, IL-15, IL-21), IL-12, IL-18, IL-27, TNF-α, IFN-α**/**β**,** IFN-γ and transforming growth factor (TGF)-β (Cooper MA et al., 2009; Parrish-Novak J et al., 2002; Wu Y et al., 2017; Zwirner NW and Domaica CI, 2010; Zwirner NW and Ziblat A, 2017).

IL-2, mainly produced by activated T cells, and **IL-15**, produced by macrophages, DC and bone marrow stroma, have several positive effects on NK cell functions, as they enhance proliferation and survival, co-stimulate cytokine production and amplify cytotoxic activity. Upon receptor engagement, their downstream signals induce the activation of JAK1/3 and STAT3/5, PI3K pathway, MAPK pathway and NF-κB (Waldmann TA, 2015). IL-2 and IL-15 share the IL-2/15Rβ (CD122) and γ_c (CD132) receptor subunits, which allow ligand binding with intermediate affinity, at nanomolar concentration. The inclusion of CD25/IL-2Rα subunit forms the high affinity heterotrimeric IL-2Rαβγ receptor, which responds to picomolar concentration of IL-2. The high affinity receptor is constitutively expressed on a subset on NK cells named $CD56^{bright}$ (vide infra), but it can be induced also on $CD56^{dim}$ NK cells, upon stimulation with IL-2, IL-15 (Pillet AH et al., 2009), or combinations of IL-15, IL-12 and IL-18 (Chase JM et al., 2012; Leong JW et al., 2014). IL-15Rα is primarily expressed on activated dendritic DC and macrophages, it has high affinity for IL-15 as a single subunit, and trans-presents bound IL-15 to the IL-2/15Rβγ_c complex on NK cells. It has been also described, after exposure to IFN-β, a cis-presentation of IL-15 by IL-15Rα to the dimeric IL-2/15Rβγ_c complex expressed on the same NK cell (Stonier SW and Schluns KS, 2010; Waldmann TA, 2015; Zanoni I et al., 2013). **IL-21** can be expressed by a large number of cell types, such T_H17, T_H2, follicular T_H cells; its receptor is composed by IL-21R (CD360) and the common γ_c chain to form the functional heterodimeric receptor that, upon ligand binding, induces JAK1/3 activation. On human NK cells, the stimulation with IL-21 increases cytolytic activity (Parrish-Novak J et al., 2000, 2002; Venkatasubramanian S et al., 2017) *in vitro* and in HIV

patients (Iannello A et al., 2010); in mice, IL-21 stimulation promotes the acquisition of functional markers and decreases their proliferation (Brady J et al., 2004).

IL-12 is produced by DC and macrophages in response to pathogens; during infection with intracellular parasites, IL-12 promotes IFN-γ production and target recognition in cooperation with other stimuli (Hashimoto W et al., 1999; Thierfelder WE et al., 1996; Vignali DAA and Kuchroo VK, 2012; Zwirner NW and Ziblat A, 2017). IL-12 receptor is composed by two subunits, IL-12R β1 and IL-12R β2; upon ligand binding it activates JAK1 and TYK2, leading to T-bet activation, crucial promoting factor for IFN-γ transcription (Trinchieri G, 2003; Zwirner NW and Ziblat A, 2017).

Another DC- and macrophage-produced cytokine important for NK activity is **IL-27**, which displays proinflammatory effects. IL-27 affects NK cell activation state, up-regulates IL-2Rα and NKp46 expression, enhances NKp46-driven cytotoxicity, and rituximab- and other therapeutic monoclonal antibody (mAb)-dependent cytotoxicity and cytokine production (Ziblat A et al., 2015; Zwirner NW and Ziblat A, 2017). IL-27 also primes NK cells for responsiveness to IL-18-induced IFN-γ production, by up-regulating T-bet (Matsuda JL et al., 2007).

IL-18 belongs to the IL-1 family, and promotes NK cell activation and effector functions, in cooperation with IL-12 (Chaix J et al., 2008). IL-18R signal transduction acts through the Toll/IL-1 receptor (TIR) domains, and triggers the binding of MyD88, phosphorylation of the Interleukin-1 receptor-associated kinases (IRAKs), and leads to the activation of NF-κB (Dinarello CA et al., 2013).

Type I IFN (IFN α and β) are innate cytokines abundantly produced during viral infections, especially by specialized plasmacytoid pDC. Their shared receptor (IFNAR) is broadly expressed, and upon ligand binding, it initiates the JAK/STAT pathway (Ivashkiv LB and Donlin LT, 2014). Type I IFNs potentiate NK cell functional activity, increase TRAIL expression and also IL-15 production by NK cells (Nguyen KB et al., 2002; Paolini R et al., 2015). Moreover, DC are themselves stimulated by type I IFN to produce IL-15, and to indirectly activate NK cells (Ivashkiv LB and Donlin LT, 2014; Lucas M et al., 2007).

TGF-β is a powerful immunosuppressive cytokine, secreted by a variety of cell types, including macrophages and regulatory T cells (T_{reg}). It is produced in an inactive form that needs processing to become active. The receptor is composed by two TGF-βRI chains and two TGF-βRII chains, and it is broadly expressed; intracellular signal propagation occurs thanks to the phosphorylation of small mother against decapentaplegic (Smad)-2 and-3 that translocate into the nucleus, activate Smad-4 transcription factor, leading to downstream gene expression (Massagué J, 2012). Moreover, in NK cells there is a Smad-independent signal transduction that involves other pathways, such as the phosphoinositide 3-kinase (PI3K)/Akt axis, leading to mammalian target of rapamycin (mTOR) inhibition. As a result, NK cells reduce their metabolism and proliferation, and decrease their functional activity (Viel S et al., 2016). Moreover, TGF-β suppresses the expression of T-bet and Gata3, crucial transcription factors in NK cell maturation (Marcoe JP et al., 2012).

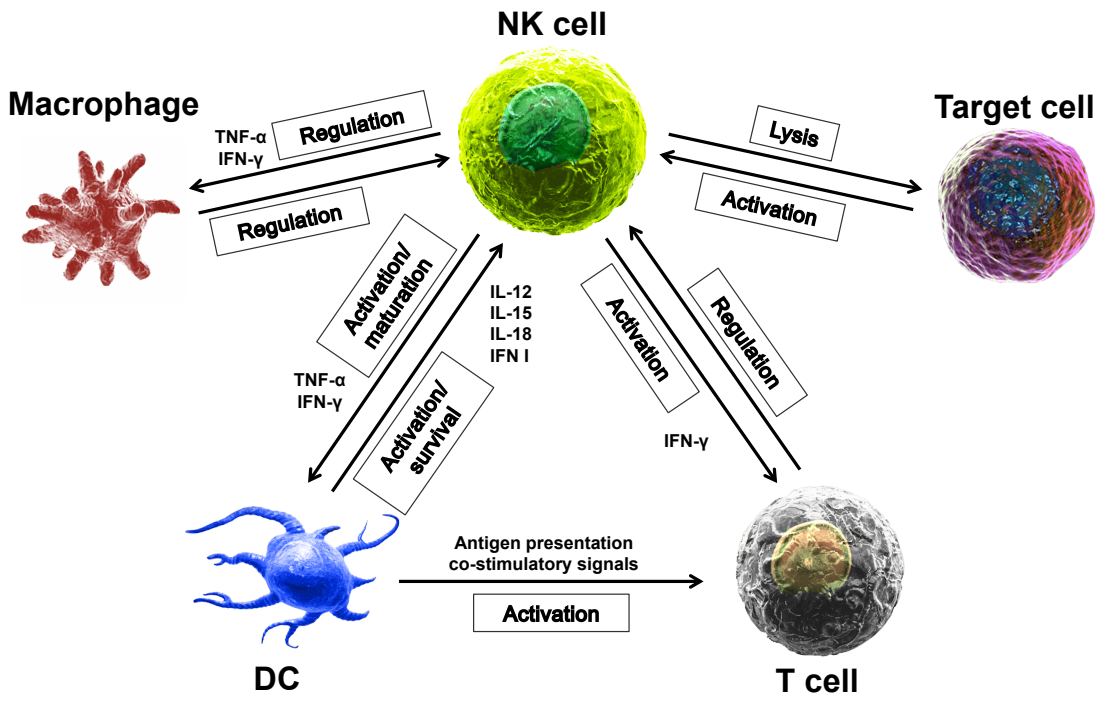

Figure 1. NK cell biological functions and cellular crosstalk

NK cells can be activated by the recognition of target cells. This leads to target cell lysis, and to the production of various cytokines and chemokines. NK cells participate in shaping the subsequent adaptive response, engaging a crosstalk with DC, macrophages and T cells. Adapted from Vivier E and Ugolini S 2010- Nature Immunology Poster https://www.nature.com/nri/posters/nkcells/nri1012_nkcells_poster.pdf

1.2 NK receptors

NK cells interact with other immune or non-immune cells through the engagement of a variety of receptors, resulting in inhibitory or activating signals, whose overall balance determines the activation state and the strength of NK cell responses, which result in target cell killing and/or immune regulation (**Figure 2**). Differently from TCR and BCR, NK cells receptors are germ-line encoded; moreover, the expression of members of the killer-cell immunoglobulin-like receptor (KIR) family is stochastic, resulting in different receptor combinations in each cell, which provide individualized target recognition capability (Guia S et al., 2018).

NK cells display several recognition strategies that allow the sensing of:

a) the levels of self-molecules expressed on normal cells, but down-modulated in infected and malignant cells. HLA molecules are indeed recognized as hallmark of “self” cells by inhibitory receptors on NK cells, blocking self-reactivity (Shifrin N et al., 2014; Yokoyama WM and Kim S, 2006);

b) the levels of self molecules that undergo overexpression in malignant and infected cells, named stress-induced ligands, that are recognized by activating receptors (Kruse PH et al., 2014; Lanier LL, 2015);

c) the presence of pathogen-derived molecules, as a result of the infection process of either bacteria and viruses (Kruse PH et al., 2014; Lanier LL, 2015).

Many NK receptors are grouped in families, based on sequence homology. In several cases, activating and inhibitory receptors belong to the same family, and often recognize shared ligands.

NK cell activation is the result of the synergistic engagement of activating receptors, in most cases. Indeed, except for CD16, none of the other activating receptors is sufficient alone to trigger cytotoxicity and cytokine production (Bryceson YT et al., 2006, 2009; Long EO et al., 2013). On the other hand, NK cell activation threshold and responsiveness to the engagement of individual activating receptors may be deeply affected by the exposure to some cytokines, such as IL-2, IL-12, IL-15, IL-18, and IL-21 (Berrien-Elliott MM et al., 2015; Bryceson YT et al., 2006). A recent paper, based on the impedance variation study, has also confirmed these results, by comparing the response triggered by selected receptors (Fasbender F and Watzl C, 2018).

Besides target recognition, NK cell receptors have also been found crucial in the

crosstalk with other immune system populations, for example with DC and macrophages. Here, NK cells recognize and kill the immature forms of these antigen-presenting cells (APC), selecting those cells able to cooperate in an efficient immune response (Malhotra A and Shanker A, 2011).

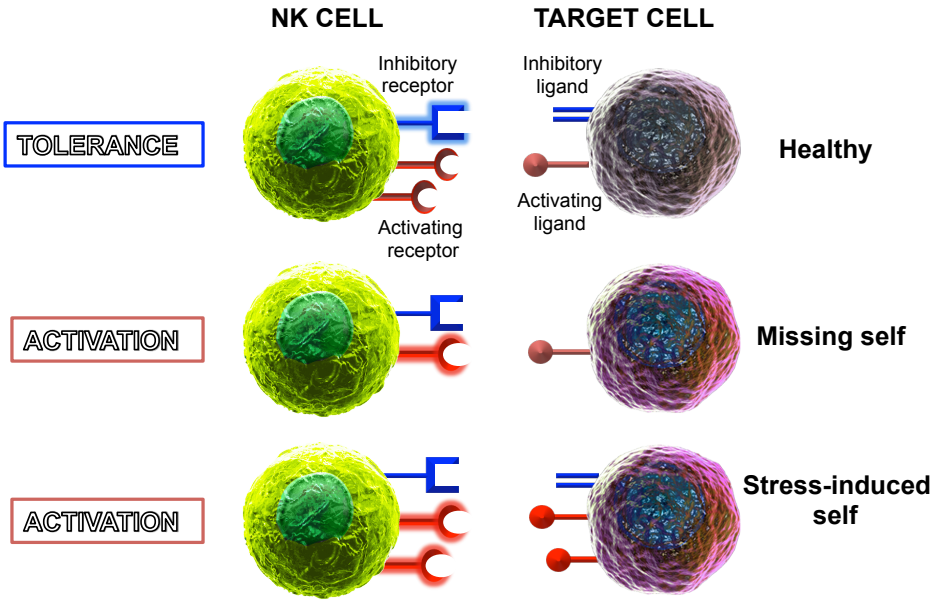

Figure 2. NK cell recognition of target cells

The integration of inhibitory (blue) and activating (red) pathways, resulting from the interaction with target cell, determines the dynamic equilibrium of NK cell activation. Adapted from Vivier E and Ugolini S 2010- Nature Immunology Poster https://www.nature.com/nri/posters/nkcells/nri1012_nkcells_poster.pdf

CD16

CD16, or FcγRIIIa, is the low affinity receptor for the Fc fragment of IgG. It is a multi-chain receptor, possessing one α-chain, responsible for ligand binding, coupled to Immunoreceptor Tyrosine-based Activation Motif (ITAM)-containing CD3ζ and FcεRIγ disulfide-linked homo- and hetero-dimers. CD3ζ has three ITAM domains, while FcεRIγ has only one, possibly resulting in a different signal transduction capability of the different CD16 complexes (Lanier LL et al., 1991). Oligomerization of CD16 complex subunits occurs thanks to the presence of charged residues with opposite sign, in the transmembrane regions of the different components (Blázquez-Moreno A et al., 2017).

ITAM motifs are found in the components of different immunoreceptor complexes, such as TCR, BCR, FcγRs, FcεRI, among others. Motif consensus sequence: D/E xxYxxL/I x-6/8 Yxx L/I is characterized by the presence of two phosphorylable tyrosines, both necessary and sufficient for the induction of downstream intracellular signals (Reth M, 1989; Romeo C et al., 1992). CD16 aggregation triggers ITAM phosphorylation by Lck and Fyn Src-family kinases. This event leads to the recruitment of SH2 domain-containing ζ -associated protein (ZAP70) and Syk tyrosine kinases. Bound ZAP70 and Syk becomes themselves substrate for Src kinases, and contribute to signal propagation by phosphorylating the transmembrane adapter molecules LAT, resulting in the recruitment and activation of different signaling molecules (Humphrey MB et al., 2005; Samelson LE et al., 1999). Among them, Grb2 leads to Ras and Erk kinase activation, which promote AP-1 transcription complex activation. In parallel, phosphorylated LAT also scaffolds and activates phosphatidyl-inositol-3-OH kinase (PI3K), phospholipase Cγ (PLCγ) and Vav-SLP-76 complex. Guanine nucleotide exchange factor Vav acts on Rac, and initiates a distinct MAP kinase cascade, while JNK activation converges on the activation of AP-1 components. Both PI3K and PLCγ use the membrane phospholipid phosphatidylinositol-4,5- bisphosphate (PIP_2) as common substrate. PI3K activity converges on Vav family proteins and Akt kinase, while PLCγ pathway contributes to ERK and NF-κB activation. All these signaling pathways lead to cytoskeletal re-organization, cytotoxic granule polarization and secretion, and gene activation (Samelson LE, 2002). In contrast to other activating receptors, CD16 aggregation by IgG-coated target cells is by itself sufficient to activate antibody-dependent cell-mediated cytotoxicity (ADCC) and cytokine production in resting human NK cells (Bryceson YT et al., 2006; Fauriat C et al., 2010a).

A genetic factor that affects CD16 responsivity is represented by an allelic dimorphism in the CD16A gene, which results in a functional aminoacidic difference (valine or phenylalanine) at position 158. In fact, the presence of valine (V) confers to CD16 receptor a higher affinity for Fc portion of IgG with respect to the phenylalanine (F)-carrying one (Koene HR et al., 1997). In that respect, individuals can be FF or VV homozygous, or VF heterozygous.

Besides the target-directed functional activity, CD16 triggering also regulates

proliferative and apoptotic stimuli in specific contexts. CD16 triggering with IgG or agonist antibodies, induces NK cell proliferation (Lee HR et al., 2017). Conversely, CD16 aggregation inhibits the proliferation induced by IL-2, partially through the induction of the apoptosis. It has been hypothesized that this mechanism helps to control and limit NK cell functionality in the late stages of immune response when the adaptive branch of immune system is finally working (Azzoni L et al., 1995; Eischen CM et al., 1996; Ortaldo JR et al., 1995; Warren HS and Kinnear BF, 1999).

CD16 expression is regulated during NK cell differentiation (see below), and is susceptible to modulation: some studies have proposed a post-transcriptional regulation of CD16 expression, through miR-218 microRNA, which negatively affects CD16 mRNA transcription (Victor AR et al., 2018). Moreover, CD16 is sensitive to disintegrin and metalloproteinase (ADAM)-10 and -17 cleavage (Pham DH et al., 2017); these proteases are induced after stimulation with IL-2 and IL15, and with tumor cells (Feldinger K et al., 2014; Romee R et al., 2013).

Natural cytotoxicity receptors (NCR)

Natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46 are a specialized group of activating receptors that mediate a key role in target cell recognition, identified in the 90s (Pende D et al., 1999; Pessino A et al., 1998; Vitale M et al.,, 1998). NCR are transmembrane proteins that belong to the immunoglobulin superfamily, and are associated with ITAM-containing accessory chains that allow surface expression and signalling capability; NKp44 pairs with DNAX-activating protein of 12kD (DAP12), while NKp30 and NKp46 interact with CD3ζ and FcεRIγ (Hudspeth K et al., 2013; Kruse PH et al., 2014; Moretta A et al., 2001).

NKp46 and NKp30 are constitutively expressed by NK cells, and in some restricted groups of T cells and ILC (Kruse PH et al., 2014). NKp44 is expressed on activated NK cells, on specialized tissue NK cell subsets, such as in the decidua, gut lamina propria, and tonsils (Cella M et al., 2009; Horton NC and Mathew PA, 2015; Takayama T et al., 2010), and also in a subset of plasmacytoid DC in the tonsils (Fuchs A et al., 2005).

NCR are crucial receptors in recognition and killing of several tumors including melanoma, carcinoma and multiple myeloma (Sun C et al., 2015), but their ligands

have not been conclusively identified yet. It has been reported that the three NCR, with different individual specificities, can recognize three different heparan sulphate glycosaminoglycans typical of tumor cells (Hecht ML et al., 2009; Kruse PH et al., 2014). Other self-ligands are HLA-B associated transcript 3 (BAT3) and Vimentin, deriving from the intracellular milieu of malignant cells, and recognized by NKp30 and NKp46, respectively (Garg A et al., 2006; Pogge von Strandmann E et al., 2007). B7-H6 (NCR3LG1), also expressed on the surface of tumor cell lines and primary tumors, is the main ligand for NKp46 (Gutierrez-Franco J et al., 2018; Horton NC and Mathew PA, 2015; Matta J et al., 2013). Recently, it has been reported by Colonna's group that platelet-derived growth factor (PDGF)-DD is a relevant ligand for NKp44. This protein is produced by many tumors and promotes the malignant transition and cell proliferation. Engagement of NKp44 by PDGF-DD stimulated the secretion of IFN-γ and TNF-α, and other proinflammatory cytokines, which in turn induced the downregulation of tumor cell-cycle genes and tumor growth arrest (Barrow AD et al., 2018). Moreover, proliferating cell nuclear antigen (PCNA), which is overexpressed by tumor cells and interacts with HLA I, can be recognized by NKp44. Interestingly, this binding is generating an inhibitory signal for the effector NK cell; indeed, NKp44 also has an active Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) motif (vide infra), able to inhibit cytotoxicity, IFN-γ release, and representing a tumor immune escape strategy (Horton NC and Mathew PA, 2015).

Several studies have demonstrated the involvement of NCR in NK cell-mediated antiviral responses (Brusilovsky M et al., 2012). NKp46 and NKp44 can directly recognize cell surface expressed pathogen-derived molecules as hemagglutinins (Orthomyxovirus) and hemagglutinin-neuraminidases (Paramyxovirus) (Arnon TI et al., 2001; Mandelboim O et al., 2001). Human cytomegalovirus (HCMV) tegument protein pp65 is recognized by NKp30, whose binding disrupts the association with CD3ζ adapter, blocking the transduction signal (Arnon TI et al., 2005).

Different NCR can cooperate, by recognizing multiple ligands on the same target cell, for optimal recognition and functional activation of effector NK cells (Long EO et al., 2013).

Killer Immunoglobulin-like Receptor (KIR) family

The KIR polygenic locus includes 15 genes and 2 pseudogenes, on chromosome 19 in the position 19q13.4. All genes code for transmembrane glycoproteins expressed on NK cells and a subset of T cells, and present either 2 or 3 Ig-like domains in the extracellular region, which allows a classification in 2D or 3D receptors, respectively. Their major ligands are HLA class I molecules. KIR ligands include C1 and C2 groups of HLA-C alleles (for 2D receptors), some HLA-A and HLA-B alleles (for 3D receptors), and HLA-G (for KIR2DL4) (Campbell KS and Purdy AK, 2011). KIR family includes highly homologous activating and inhibitory members, that differ for the sequence and the length of the cytoplasmic domains (Thielens A et al., 2012).

Inhibitory KIR (iKIR) display a longer cytoplasmic tail (identified as L), which includes an ITIM motif; this is characterized by V/I/LxYxxL/V sequence whose tyrosines, upon ligand recognition, undergo phosphorylation by SRC family kinases, and promote recruitment of SHP-1 and SHP-2 phosphatases that block the propagation of activating signals (Campbell KS and Purdy AK, 2011; Long EO, 2008; Long EO et al., 2013).

Activating KIR (aKIR) have a short truncated cytoplasmic tail (identified as S), and associate with DAP-12 adapter, except for KIR2DL4 that couples with FcεRIγ chain (Kikuchi-Maki A et al., 2005). Despite the high homology of their extracellular domains with iKIR, the identification of aKIR ligands remains in most cases elusive; only for some of them, the recognition of HLA-C2, -C1 and A alleles has been demonstrated (Della Chiesa M et al., 2015; Varbanova V et al., 2016).

The genetic asset of the KIR locus is highly complex. The repertoire of KIR genes inherited by an individual represents a haplotype. Two types of haplotypes can be distinguished: type A, with a fixed number of inhibitory receptors and few activating receptors, and type B, with a variable number of inhibitory receptors and several activating members; KIR3DL3, KIR2DL4 and KIR3DL2 genes are common to all haplotypes. Moreover, KIR genes show an extensive polymorphism (Parham P, 2005; Uhrberg M et al., 1997; Vilches C and Parham P, 2002). Expression of inherited KIR genes occurs stochastically during the differentiation process of individual NK cell precursors (see below), generating a high number of different mature effectors, expressing different KIR combinations (more than 10^4) (Horowitz

A et al., 2013; Parham P, 2005; Vierra-Green C et al., 2012).

The physiological role of iKIR interaction with self-HLA class I molecules is to provide a mechanism that protects healthy self cells from potential NK cell self-reactivity. In selected pathological conditions, i.e. infection by several viruses, or tumor progression, HLA class I down-modulation or loss may occur, as an immunoevasion strategy to avoid recognition by CTL. Diminution of HLA class I expression, named “missing self”, lowers NK cell activation threshold, as the missing engagement of iKIR conveys weakened inhibitory signals (Yokoyama WM and Kim S, 2006; Shifrin N et al., 2014).

Noteworthy, some studies described a lower affinity of aKIR with respect to iKIR for HLA class I molecules (Ivarsson MA et al., 2014; Moesta AK and Parham P, 2012), and it has been proposed that aKIR recognition of HLA molecules may be highly dependent on viral peptides, or altered self proteins, allowing them to actively participate to target cell recognition (Ivarsson MA et al., 2014).

Several reports have shown a significant association between certain KIR haplotypes and the susceptibility to some viral infections, some tumors and autoimmune diseases, suggesting a role for KIR in their onset/progression (Boyton RJ and Altmann DM, 2007; Falco M et al., 2013).

LIR

LIR family members are transmembrane proteins, widely expressed in hematopoietic-lineage cells and interact with HLA molecules. They are encoded by 13 genes; among them, members classified as LIRB has inhibitory effects, and just two of them are expressed on NK cells, LIRB1 and LIRB5.

LIRB display 2 or 4 extracellular Ig-like domains, and a long ITIM-containing cytoplasmic tail. LIRB1 is implicated in the recognition of HLA-I and of the human HCMV HLA class I homolog UL18 protein (Cosman D et al., 1997). HCMV-driven expression of UL18 by the infected cells is probably a viral immune escape strategy, to avoid NK cell-mediated recognition and killing of the infected cell (Prod’homme V et al., 2007). Moreover, some LIR gene polymorphisms have been associated with autoimmune disease predisposition (Zhang J et al., 2017).

C-type lectin receptors

The genes coding for several NK receptors that belong to the C-lectin superfamily are clustered in the Killer Lectin-like Receptor (KLR), on human chromosome 12. The C-lectin superfamily cluster members have different roles and ligand specificities (Bartel Y et al., 2013; Seliger B et al., 2016).

NKG2A and NKG2C heterodimerize with CD94, to form an inhibitory and an activating receptor, respectively, specific for HLA-E, a non-canonical HLA class I molecule. CD94/NKG2A provides the inhibitory signal through its intracellular tail that contains two ITIM domains, while CD94/NKG2C couples to DAP12 to provide activating signals. Their expression is regulated during NK cell differentiation, as CD94/NKG2A is expressed earlier during the maturation pathway, while it is less expressed on the terminal stage, in parallel with the progressive acquisition of NKG2C (Stabile H et al., 2018).

HLA-E molecules are constitutively expressed on the cell surface at low level, but may undergo up-regulation in stressed and malignant cells. HLA-E stabilization on the cell membrane requires loading with self and non self peptides, such as heat shock protein 60 (hsp60)-derived peptide (Michaëlsson J et al., 2002), the leader peptides derived from classical HLA class I A, B, and C, hence representing the overall expression of HLA molecules on the cell surface (Borrego F et al., 2005), and peptides from the HCMV encoded protein UL40 (Ulbrecht M et al., 2000). Although it has not been definitively understood how HLA-E-associated peptides affect recognition of CD94/NKG2A and CD94/NKG2C, it has been shown in different contexts that HCMV infection accelerates NK cell terminal differentiation, and promotes the expansion of $NKG2C^{bright}$ NK cells (Gumá M et al., 2004; Hammer Q and Romagnani C, 2017).

NKG2D was initially identified in 1991 by Houchins and colleagues (Houchins JP et al., 1991), but its role has been ignored until 1999, when MIC (MHC class I chain related) A, one of its ligands, was identified (Bauer S et al., 1999). NKG2D homodimers are expressed on NK cells, but also on $\gamma\delta TCR^{+}$ and on $CD8^{+}$ T cells, and on some $CD4^{+}$ T cells (Lanier LL, 2015).

NKG2D recognizes multiple ligands that are structurally related to HLA I molecules and are expressed by stressed, infected, and transformed cells. These are MICA, MICB and UL16-binding proteins (ULBP)-1-6 (Dhar P and Wu JD, 2018).

After ligand recognition, NKG2D signalling ability depends on the association with DAP10 adapter protein. It carries a YINM motif, leading to an ITAM-independent pathway that, upon ligand-triggered tyrosine phosphorylation, allows the binding of PI3K, PLCγ and Grb2. Grb2 mediates the recruitment of Vav proteins (Lanier LL, 2015). NKG2D engagement alone can induce chemokine production and release, while cytotoxic activity, instead, needs a synergy with other receptors (Bryceson YT et al., 2006; Fauriat C et al., 2010a; Lanier LL, 2015). NKG2D receptor thus represents an important tool for NK cell-mediated protection against pathogens and tumors. Indeed, NKG2D ligand shedding from tumor cell surface, either by protease cleavage, or by exosome release, is considered a mechanism of tumor evasion (Lanier LL, 2015). Moreover, HCMV displays several strategies to interfere with NKG2D ligand expression on infected cells (Dunn C et al., 2003; Lanier LL, 2015; Slavuljica I et al., 2011).

DNAM-1 family receptors

The DNAX accessory molecule-1 (DNAM-1) family comprises four recognized members: DNAM-1 (CD226), T cell Ig and ITIM domain (TIGIT, Vstm3) T cell activation, increased late expression (TACTILE, CD96), and CRTAM (CD355), all belonging to the Ig superfamily.

DNAM-1 is expressed on almost the totality of NK cells, on a fraction of $CD8^+$ and $CD4^+$ T cells, and on some myeloid cells (De Andrade LF et al., 2014). It has 3 putative phosphorylation sites in the intracellular domain that are phosphorylated upon ligand binding. The association with Lymphocyte function-associated antigen 1 (LFA-1) is necessary for the signal transduction, indeed, it recruits Fyn SRC kinase to phosphorylate the other tyrosines in DNAM-1 and to initiate DNAM-1 downstream signalling. The cooperation with other activating signals is essential to promote NK activation (De Andrade LF et al., 2014).

TIGIT is an inhibitory receptor expressed on NK cells, on memory T cells and T_{reg} and on $CD4^+$ T cells upon activation. The percentage of $TIGIT^+$ NK cells can vary among individuals, and also among cells of the same individual, where its intensity inversely correlates with NK cell functional ability (Wang F et al., 2015). It has one ITIM domain and an immunoglobulin tail tyrosine (ITT)-like motif in the intracellular portion, both actively working in decreasing cell activating status and functional

ability (Liu S et al., 2013; Stanietsky N et al., 2013).

DNAM-1 and TIGIT have two shared ligands, poliovirus receptor (PVR) and Nectin-2, widely expressed on epithelial cells and in immune system populations under physiological conditions. These ligands are stress-induced, hence increased, during cell transformation and viral infections, and also upon activation of hemopoietic cells (Cerboni C et al., 2014). TIGIT binds PVR with high affinity, and competes with DNAM-1, contributing to the inhibition of NK cell functionality (Levin SD et al., 2011; Stanietsky N et al., 2013). It is also important in the crosstalk between immune cell populations, as it has been recently demonstrated that NK cells can be suppressed in their degranulation potential by PVR-expressing myeloid-derived suppressor cells (MDSC), through TIGIT engagement (Sarhan D et al., 2016).

Others not extensively studied members of the family are TACTILE and CRTAM. TACTILE is expressed on all NK cells, while CRTAM is confined on activated NK cells. Both can be also expressed on $CD4^+$ and on $CD8^+$ T cells. Their ligands are stress-induced ligands, respectively PVR and Necl-2. In human NK, their activating role in NK function, has been described (Chan CJ et al., 2014; Dessarthe B et al., 2013; Fuchs A and Colonna M, 2006; De Andrade LF et al., 2014)

PD-1

Several MHC class I-specific and non-specific inhibitory receptors are expressed on human NK cells and restrain their functional response. Among them, and in addition to inhibitory KIR, inhibitory member of DNAM-1 family, TIM-3 and LAG-3 (Anderson AC et al., 2016), there is Programmed Death-1 (PD-1). PD-1 is a receptor crucial in the suppression of the immune response, also expressed on activated T cells, and is especially involved in the tumor immune escape triggered by the engagement of its ligands, PD-L1 and PD-L2, which are expressed by malignant cells and activated immune cells. PD-1 possesses an ITIM and an immunoreceptor tyrosine-based switch motif (ITSM) domain. In T cells, both can associate with SHP phosphatases and block activation through the suppression of PI3K/AKT signaling; in NK evidence of a similar signaling are reported (Bardhan K et al., 2016; Liu LL et al., 2017; Sharpe AH and Pauken KE, 2017).

PD-1 has been recently has been identified on $CD56^{dim}$ subset of NK cells, in around 1/4 of healthy subjects, and its presence is restricted to $HCMV^+$ donors (Della Chiesa M et al., 2016; Pesce S et al., 2017). $PD1^+$ NK cells have been found enriched in the tumor microenvironment. From the functional point of view, $PD\text{-}1^+$ NK cells are hypofunctional and hyporesponsive in terms of tumor cell killing, and this effect can be reverted by using anti-PD-1 blocking antibodies (Della Chiesa M et al., 2016; Liu LL et al., 2017).

Pattern-recognition receptors (PRR)

Innate immune cells express Pattern-recognition receptors (PRR) specifically able to recognize recurrent motifs carried by viruses, bacteria and fungi, named pathogens-associated molecular patterns (PAMP), and also involved in the recognition of damage-associated Molecular Patterns (DAMP) (Mogensen TH, 2009; Tang D et al., 2012). NK cells express only some Toll-like Receptors (TLR), such as TLR2, TLR3 and TLR5. Moreover, as mentioned before, the capability of some NCR to directly recognize microbial ligands has been reported. TLR engagement leads to IRFs and NF-κB activation, resulting in type I IFN and other proinflammatory cytokine production. Moreover, they can co-operate with receptors belonging to other classes to induce a more efficient NK cell activation (Adib-Conquy M et al., 2014; Sivori S et al., 2014)

1.3 NK development and differentiation

NK cells development occurs in the bone marrow from a $CD34^+$ hematopoietic stem cells (HSCs), in a common pathway that originates all Innate Lymphoid Cell (ILC) subsets; however, several studies report that NK cells can further differentiate in tissues, especially in secondary lymphoid organs, liver, uterus and thymus (Fauriat C et al., 2010b). Human NK cells differentiation is a not completely understood multistep process, where some markers are acquired and in parallel some others are lost (**Figure 3**). This process results in the progressive restriction toward the NK cell lineage and acquisition of recognition repertoire and functional competence (Freud AG and Caligiuri MA, 2006).

Stage 1 progenitors are $CD34^+CD117^+CD123^{+/-}FLT3L^+$. This stage corresponds to

the common lymphoid progenitor (CLP), which has the potential to give rise to B, T, NK, ILCs and DCs. Stage 1 progenitors have been found in cord and adult blood, in bone marrow, in several adult tissues and in fetal liver. Interestingly, from this progenitor all the ILC types can originate, through a recently identified intermediate stage that is $Lin^{-}CD7^{+}CD127^{+}CD117^{+}$. The differentiation program is influenced by the microenvironment as required, under the stimulation of different cytokines, at every age and in every tissue, to replenish tissue resident ILC pools, including NK cells (Lim AI et al., 2017).

Stage 2, or NK progenitors (NKP) are $CD34^{+}CD117^{+}CD123^{-}FLT3L^{+}CD127^{+}$ and are lineage-restricted. A key step is the acquisition of the IL-2/IL-15Rβ chain (CD122), whose expression is induced by stem cell factor (SCF) and FLT-3, confers responsivity to IL-15 stimulation and allows further differentiation of NK cells, which is dependent on the action of Eomes and Tbx21 transcription factors (Scoville SD et al., 2017).

Stage 3, or immature NK cells are: $CD34^{-}CD117^{+}CD123^{-}$ $CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$ (Stabile H et al., 2018). Despite they are committed to be NK cells, they are not yet endowed with the ability to perform the two hallmark functions of NK cells: degranulation and IFN-γ production (Freud AG and Caligiuri MA, 2006).

Stage 4 cells are identified as $CD34^{-}CD117^{+/-}CD94^{+}CD16^{-}$. They also express CD56 at high levels, hence defined $CD56^{bright}$ NK cells (Stabile H et al., 2018). CD56, or Neural Cell Adhesion Molecule (N-CAM), is a member of the Ig superfamily and represents an important marker for NK cells. Its functional role on NK cells has not been definitively assessed yet, although it has been reported to mediate homotypic adhesion, to bind fibroblast growth factor, and to mediate direct recognition of some microorganisms, as the *Aspergillus fumigatus,* resulting in the activation of the NK cell (Van Acker HH et al., 2017; Ziegler S et al., 2017). $CD56^{bright}$ NK cells mainly localize in secondary lymphoid organs, while they represent only 10% of NK cells in the peripheral blood and in the liver. $CD56^{bright}$ are preferentially $CD94/NKG2A^{+}CD94/NKG2C^{-}CD57^{-}KIR^{-}CD16^{dim}$, they proliferate in response to cytokines, and produce large amounts of IFN-γ and TNF-α, but they have a low cytotoxic potential and low ability to perform ADCC. The $CD56^{bright}$ subset is believed to precede the final stage of NK differentiation (Freud AG et al.,

2006; Luetke-Eversloh M et al., 2013).

Indeed, stage 5, or $CD56^{dim}$, represent the majority of peripheral blood NK cells, express CD16 receptor at high density, and are thought to represent the final stage of NK cell differentiation. They also typically display CD16 at high levels ($CD16^{bright}$), are CD94/NKG2A^{low}CD94/NKG2C^{+}CD57^{+}KIR^{+}, and show low proliferative capability. Historically, the functional activity of these cells was thought to be confined to their high cytotoxic potential, while more recently it has been shown that they can also produce a large amount of cytokines (Cooper MA et al., 2001b; Luetke-Eversloh M et al., 2013; Montaldo E et al., 2013).

The unique milieu available in different peripheral tissues might influence *in situ* differentiation of resident NK cells, and could contribute to explain the heterogeneity of NK cell subsets (Freud AG and Caligiuri MA, 2006). Even in the same tissue, heterogeneity of NK cells is observed, reflecting different stages of differentiation. The tissue distribution and homing ability of NK cells depend on their expression of chemokine receptors (Freud AG and Caligiuri MA, 2006). $CD56^{dim}$ cells express chemerin-R, CXCR1 and CX3CR1, which determine their ability to migrate in inflamed tissues. Differently, $CD56^{bright}$ express CCR7 and CD62L, and are attracted to secondary lymphoid organs (Bernardini G et al., 2013; Del Zotto G et al., 2017; Griffith JW et al., 2014; Soriani A et al., 2018).

NK cell functional competence is acquired during differentiation, in a still partially undefined process, named education (Boudreau JE and Hsu KC, 2018a). It has been established that the acquisition of full competence requires the contact of stochastically expressed KIR inhibitory receptors with self-HLA molecules, during bone marrow differentiation. In parallel, this mechanism provides a protection of self cells from NK cell attack. NK cells without any inhibitory receptors are hypofunctional, thus preventing self-reactivity (Boudreau JE and Hsu KC, 2018b; Fauriat C et al., 2010b; Goodridge JP et al., 2015).

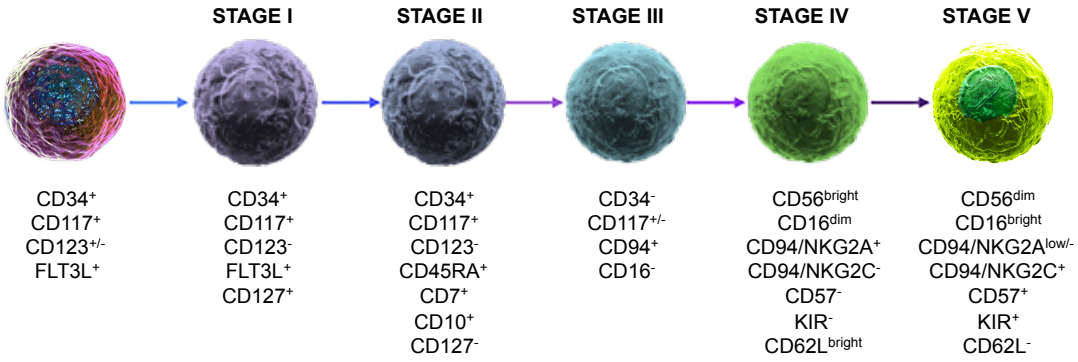

Figure 3. Stages of human NK cell differentiation

The multiple-stage model of NK cell development is based on the sequential acquisition of activating/inhibitory receptors, adhesion molecules, and by the progressive functional maturation. Five discrete stages have been described, on the basis of characteristic arrangement of cell surface receptors. Modified from Stabile H et al., 2018.

2. NK CELLS AND TUMORS

NK cells are relevant players in the anti-tumor response for their ability to interact with the tumor directly, by recognizing and lysing tumor cells, and indirectly, by orchestrating the immune response in the tumor microenvironment. For their important role, NK cell have been exploited in anti-tumor therapies in multiple strategies and successful approaches (Fang F et al., 2017).

2.1 NK cells in anti-tumor immune response

NK cells provide a first line of defence against tumor development and metastasis spreading, thanks to their ability to recognize and become activated upon recognition of transformed cells (Gajewski TF et al., 2013). Historically, NK cells have been recognized as outstanding contributors in the control of haematological tumors (Baier C et al., 2013; Farnault L et al., 2012); lately, their potential in solid tumor infiltration and killing has also been considered (Stojanovic A and Cerwenka A, 2011). Interestingly, increasing evidences also report the NK cell contribution to the killing of tumor stem cells, and hence in preventing tumor relapse (Luna JI et al., 2017; Tallerico R et al., 2013, 2016).

In parallel with the multistep acquisition of mutations, epigenetic alterations, and metabolic imbalance, stress-induced ligands are also up-regulated on tumor cells, which are recognized by several NK activating receptors. For example, MICA, MICB and ULBP 1-6 trigger NKG2D activation, B7-H6 and BAT3 are recognized by NKp30, PVR and Nectin-2 interact with DNAM-1 activating receptor (Marcus A et al., 2014; Waldhauer I and Steinle A, 2008). Moreover, tumor variants expressing lower or absent levels of HLA class I molecules, which emerge as a result of immune pressure exerted by CTL, are more sensitive to NK cell recognition, thanks to loss of the inhibitory brake provided by HLA I-specific inhibitory receptors (Garrido F et al., 1997; Kageshita T et al., 1999).

NK cells not only directly contribute to the recognition and killing of tumor cells, but also enhance the effector function of other immune subsets, by exerting immunomodulatory activities. This is mainly accomplished by the capability of NK cells to secrete cytokines and chemokines. The development of M1 macrophages requires IFN-γ, produced in large amounts by activated NK cells. M1

macrophage presence at tumor site promotes tumor regression via the activation of T_H1 response and by the secretion of nitric oxide (Aqbi HF et al., 2018). They also produce IL-12 that stimulates NK cells to produce IFN-γ and to up-regulate CD25/IL-2R*α* expression (Duggan MC et al., 2018).

Another innate immune subset recruited and activated by NK cells is the DC. They promote antigen-specific T cell activation, and contribute to the orchestration of anti-tumor adaptive response. Their presence in the tumor microenvironment is a positive prognostic factor, and is also associated with an increased number of infiltrating T cells (Chen DS and Mellman I, 2013; Lee SC et al., 2014; Spranger S et al., 2017). NK cell-secreted chemoattractants, including CCL5 and CXCL1/2, are necessary for DC recruitment (Böttcher JP et al., 2018). NK-produced IFN-γ induces DC maturation and cross-presentation, and T cell priming (Martín-Fontecha A et al., 2004; Walzer T et al., 2005; Wittrup KD, 2017). In parallel, IL-12 and type I IFN produced by DC contribute to NK activation and boost their IFN-γ production and cytotoxicity (Walzer T et al., 2005). NK-DC cell-cell contacts are also necessary, such as IL-15 trans-presentation, and the interactions between other receptor/ligand couples. NKp30 plays a central role in the NK/DC crosstalk. The maturation of monocyte-derived DC is induced by NKp30 triggering, and to the subsequent release of TNF-α and IFN-γ by NK cells. Moreover, NK cells kill immature, but not mature, DC in a NKp30-dependent way (Moretta A, 2005; Walzer T et al., 2005; Wehner R et al., 2011).

Nevertheless, many tumors evolve under the pressure of the immune response, selecting clones that can resist/evade NK cell recognition and effector capabilities (Hanahan D and Weinberg RA, 2011; Vinay DS et al., 2015). For example, it has been demonstrated in animal models, and confirmed in patients, that some tumor aggressive variants lose the expression or shed the ligands for NKG2D activating receptor. This last strategy, moreover, impairs the immune system also in other locations, far from the tumor site (Dhar P and Wu JD, 2018; Marcus A et al., 2014; Zhang J et al., 2015). To hamper NK cell activation, the tumor secretes immunosuppressive soluble factors, such as TGF-β (Massagué J, 2012), that decreases NK cell IFN-γ production, proliferation and cytotoxicity, through mTOR repression (Viel S et al., 2016), and ADCC, through SMAD3

activation and T-bet repression (Trotta R et al., 2008).

Beside TGF-β, tumor cells also secrete other immunomodulatory factors, including IL-6, IL-10, VEGF and indoleamine 2,3-dioxygenase (IDO). These factors contribute to the generation and maintenance of tolerogenic immune subsets (Vinay DS et al., 2015). A second strategy is the recruitment of T_{reg} or myeloid derived suppressor cells (MDSC). T_{reg}, upon stimulation through their TCR, produce immunosuppressive cytokines such as TGF-β and IL-10. Further, via their high expression of CD25 they sequester IL-2, blocking NK cell and CTL activation and survival. Moreover they express inhibitory molecules such as CTLA-4 or PD-1, able to inhibit other immune populations (Beatty GL and Gladney WL, 2015; Chaudhary B and Elkord E, 2016). MDSCs produce nitric oxide and ROS, increasing inflammation, tissue damage and apoptosis of T cells. They produce anti-inflammatory cytokines, which promote T_{reg} formation, they block T cell recruitment by decreasing L- and E- selectin expression, and inhibit T cell activation by expressing PD-L1 (Parker KH et al., 2015). MDSC interfere with NK activation, induce the down-modulation of NK activating receptors and the decrease of perforin production (Elkabets M et al., 2010; Liu C et al., 2007; Mao Y et al., 2014).

Tumor-derived prostaglandin E2 (PGE2) interferes with NK cell and DC contribution to anti-tumor immunity by impairing NK cell viability and chemokine production, as well as by causing downregulation of CCL5 and CXCL1/2 receptors on DC (Böttcher JP et al., 2018). In addition, PGE2 and IDO also down-modulate tumor-expressed ligands of NK cell activating receptors, contributing to immune escape (Pietra G et al., 2012). NK cells also interact with tumor-associated neutrophils, which can be polarized toward the alternative N2 phenotype, thus promoting tumor progression (Fridlender ZG et al., 2009; Shaul ME et al., 2016). Finally, as IFN-γ induces vascular endothelial growth factor (VEGF) production, it can positively contribute to tumor growth and neoangiogenesis (Ogura K et al., 2018).

2.2 NK cell-based cancer therapies

Due to the key role of NK cells in cancer recognition and killing, an increasing

number of studies have focused on NK exploitation in cancer therapy. These strategies include the *in vivo* administration of NK-activating cytokines, or of monoclonal antibodies to manipulate the balance between activating and inhibitory receptor signalling, and the adoptive transfer of selected and/or *in vitro*-manipulated NK cell populations.

Cytokine-based therapies

For their well-known effect on NK proliferation and activation, IL-2 and IL-15 have been used in cancer therapy. IL-2 was one of the first approved cytokines for the treatment of metastatic renal cell carcinoma and metastatic melanoma. This treatment gave long lasting remission only in a fraction of patients. Moreover IL-2 high dose administration has many side effects, as capillary leakage and organ injuries (García-Martínez E et al., 2018; Sim GC and Radvanyi L, 2014). IL-2 infusion has not only effects on NK cells, but also on T cells, and on T_{reg}, which display high levels of IL-2Rα/CD25 (García-Martínez E et al., 2018). IL-2 mutants have also been created to increase the benefits of IL-2 administration. One of them, for example, is the *superkine* that displays higher agonist functions and less side effects by increasing its affinity for the IL-2Rβγ (Jiang T et al., 2016).

In contrast to IL-2, IL-15 does not promote T_{reg} activation. The intravenous administration of IL-15 induces the amplification of the NK subset, without all the side effects induced by IL-2. Several clinical studies are verifying the effect of IL-15 as a monotherapy and in combination with other chemotherapeutics and NK cell adoptive transfer (Evans R et al., 1997; Robinson TO and Schluns KS, 2017). Since IL-15 also enhances the ADCC of NK cells, its efficacy has also been experimented in association with therapeutic tumor-targeting monoclonal antibodies, such as cetuximab (anti-EGFR) and rituximab (anti-CD20) (Roberti MP et al., 2012; Robinson TO and Schluns KS, 2017; Yu P et al., 2010).

Because of the short half-life of IL-15, super-agonist compounds composed by IL-15 bound to its receptor have been produced, to increase its half-life. Improved molecules include two IL-15/IL-15Rα complexes, fused to IgG Fc portion, to increase functionality and stability (Robinson TO and Schluns KS, 2017).

IL-12, important to increase NK cell functional activities, particularly IFN-γ production and cytotoxicity, was tested for cancer therapy but suspended because

of toxicity due to high dose administration (Gokhale MS et al., 2014; Gollob JA et al., 2000, 2003).

Antibody-based therapies

Considering the power of ADCC, the idea of exploiting it to implement new effective strategies was developed, such as the driving of NK cell activity against cancer cells through tumor-targeting therapeutic mAb. For instance: anti-CD20 mAb, used in therapy of B cell malignancies and of some autoimmune diseases; anti-CD38 mAb, for the treatment of multiple myeloma and chronic lymphocytic leukaemia; anti-Her2 mAb, for invasive breast cancer; anti-CD133 mAb, for acute myeloid leukaemia (Albanell J et al., 2003; Scott AM et al., 2012a; Smith MR, 2003; Weiner GJ, 2010).

The ability of NK cells to recognize mAb-bound targets is crucial for ADCC triggering. Variations in the affinity of CD16 for antibody Fc region can make the difference. As previously mentioned, two main allelic forms of CD16 are present in the population, characterized by different binding affinity (Koene HR et al., 1997); the correlation between CD16 functional polymorphisms and clinical outcome of mAb-based therapy has been reported, even if with conflicting results (Bibeau F et al., 2009; Cartron G et al., 2002; Dall'Ozzo S et al., 2004; Farag SS et al., 2003; Weng WK and Levy R, 2003).

Besides ADCC, tumor-targeting mAb can induce other responses that promote tumor cell killing, such as complement-mediated target lysis and phagocytosis (Gajewski TF et al., 2013; Pincetic A et al., 2014; Scott AM et al., 2012b). Tumor-specific antibodies, moreover, can induce long-lasting protective effects in the treated patient by enhancing the development of adaptive immune response; this phenomenon has been called vaccinal effect, and consists in the fact that tumor-targeting mAb favour the uptake of tumor antigens by DC, and promote the generation of DAMP and a proinflammatory environment, that stimulate DC maturation and activation (Abes R et al., 2010; Pincetic A et al., 2014; Wittrup KD, 2017). NK cells are crucial promoting components of this process, as they induce DC maturation through the secretion of IFN-γ and TNF-α and by cell-cell contacts (Walzer T et al., 2005). Activated and mature DC then allow the formation of efficient and long-lasting T cell-mediated anti-tumor responses (Martín-Fontecha A et al., 2004) (**Figure 4**).

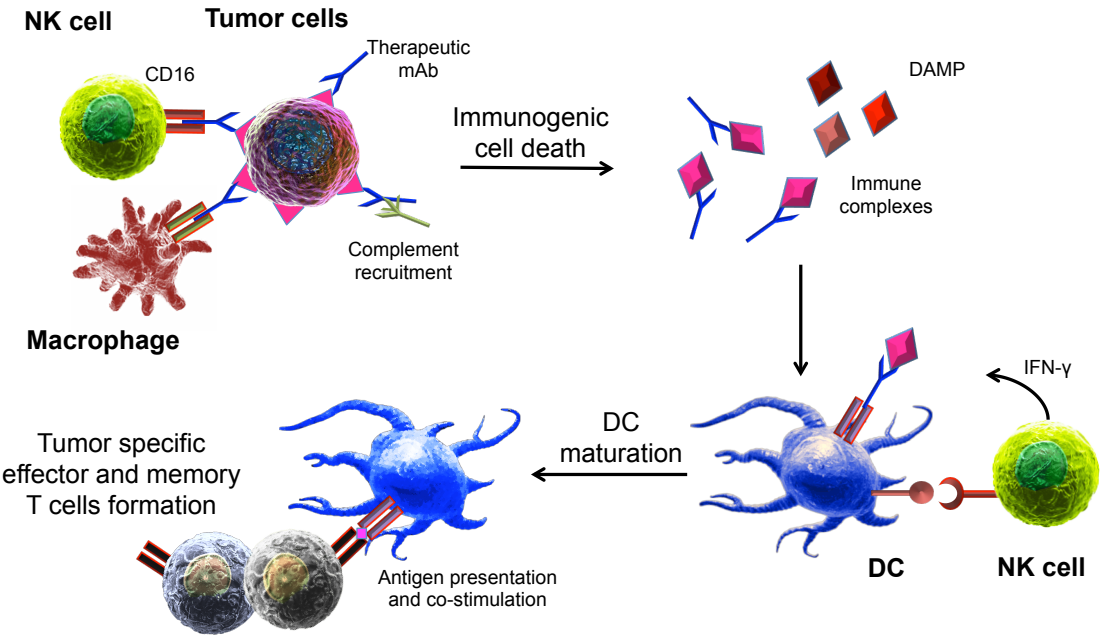

Figure 4. Model for the development of anti-tumor vaccinal effect

Therapeutic mAb induce tumor cell death by ADCC, phagocytosis and complement-induced cytotoxicity. Resulting immune complexes (antigen-containing debris+ mAb) and damage-associated molecular patterns (DAMP) can be captured and recognized by immature DC leading to their maturation and consequent expansion of tumor-specific T cells. DC activation can be strengthened by NK cell-derived IFN-γ and by cell-cell interactions. In parallel, DC-produced IL-12 results in an increased function of NK cells. The formation of a tumor-specific memory T cell pool guarantees the long term protection against tumor recurrence.

In particular, therapeutic mAb directed against CD20 antigen were the first to demonstrate clinical efficacy. CD20 is an antigen widely expressed on the surface of B lymphocytes in all stages of development, from pre-B precursor up to mature B cell, and also in several B cell malignancies. CD20-targeting mAb are widely studied and developed (Salles G et al., 2017; Weiner GJ, 2010). In 1997 the Food and Drug Administration (FDA) approved rituximab (RTX) anti-CD20 chimeric mAb for the treatment of refractory and relapsing non-Hodgkin lymphoma. In the last 20 years, RTX was used effectively also in many other B cell malignancies and in some autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, haemolytic anaemia (Gürcan HM et al., 2009; Salles G et al., 2017). In combination with chemotherapeutic drugs, it gives effective results on progression-

free and overall survival of patients with diffuse large B-cell lymphoma (DLBCL) or follicular lymphoma (Harrison AM et al., 2014; Tilly H and Zelenetz A, 2008). Upon RTX binding, low-level direct death of the target cell is induced, while ADCC, phagocytosis and complement-mediated death are triggered (Mossner E et al., 2010).

Several mechanisms of resistance to RTX have been recognized that limit its therapeutic efficacy (Freeman CL and Sehn LH, 2018); this knowledge led to the development of second-generation anti-CD20 mAb. Obinutuzumab (GA101) is a glycoengineered humanized anti-CD20 mAb that more powerfully induces direct cell death, independently from caspase activation. *Ex vivo* studies, however, suggest that this is not the principal mechanism through which the antibody is working *in vivo* (Bologna L et al., 2011; Tobinai K et al., 2017). In fact, thanks to its defucosylated Fc domain, GA101, although inducing minimal complement-mediated death, is recognized with higher affinity by CD16, improving both ADCC and phagocytosis (Bologna L et al., 2011; Mossner E et al., 2010). Its improved efficacy has been demonstrated in untreated follicular lymphoma and CLL, in combination with chemotherapy. Tumor histological subtype, host immune integrity, as well as CD16 polymorphisms may represent important factors for the preference of one mAb over the other (Freeman CL and Sehn LH, 2018).

mAb may also act with other therapeutic mechanisms, for example by targeting NK receptors that regulate their functional activities. Some examples are: anti-NKG2D, anti-CD137, anti-NKG2A, anti-PD-1 (Carotta S, 2016; Chester C et al., 2015; Kohrt HE et al., 2011, 2014; Vadstrup K and Bendtsen F, 2017; Battella S et al., 2016). A mAb that blocks iKIR was tested until phase I trial, however with disappointing results. Indeed, mAb-iKIR interaction blocked the NK education process, thus hampering their functional maturation (Benson DMJ et al., 2012; Vey N et al., 2012).

The scientific community is now investigating the efficacy of bi- and tri-specific antibodies, made to increase the effectiveness of mAb-based treatments. For instance, the CD16xCD33 bi-specific (BiKe) construct engages CD16 and CD33, resulting in an increased NK cell function against $CD33^+$ tumor and MDSC targets (Gleason MK et al., 2014). New generation TriKe is based on the addition of IL-15 to the CD16xCD33 BiKe, to increase the stimulation and the effector functions of

NK cells (Tay SS et al., 2016; Vallera DA et al., 2016). The AFM13 is an engineered construct that includes binding sites for CD16 and CD30. It has been tested alone and in combination strategy in clinical trials for Hodgkin lymphoma and in other $CD30^+$ lymphomas (Rothe A et al., 2015; Wu J et al., 2015). *In vitro* tests suggest its enhanced capability to induce NK effector functions, also after a subsequent restimulation with tumor target and cytokines (Pahl JHW et al., 2018).

NK cell adoptive transfer therapies

Considering that NK cells may provide a front line anti-tumor response, the idea of transferring NK cell into patients has been considered a possible strategy to achieve patients' remission.

First attempts were done using autologous peripheral blood NK cells that were *in vitro* treated with IL-2, to induce the generation of lymphokine-activated killer (LAK) cells, and subsequently infused back in patients, with the addition of IL-2, to increase their survival and proliferation. But only limited effects were recorded (Foreman NK et al., 1993; Jondal M and Pross H, 1975). Other groups tried to stimulate autologous NK cells with cytokines, such as IL-2, IL-15, IL-21, IL-18, or type I IFN, to increase activation markers and functional activity. Despite the promising results of *in vitro* studies, only limited effects were observed *in vivo* (Davis ZB et al., 2015; Farag SS and Caligiuri MA, 2004). Indeed, the education and maturation of such cells occurs in an immunosuppressive environment *in vivo,* and their functionality can be impaired even after *in vitro* cytokine stimulation (Burns LJ et al., 2003). For this reason, NK from healthy donors can be exploited in allogeneic adoptive transfer. Moreover, the partial mismatch between donor KIR repertoire and recipient HLA haplotype favours the NK-dependent clearance of malignant cells that do not have an altered HLA I expression (Fournier C et al., 2017; Miller JS et al., 2005; Ruggeri L et al., 2005).

NK cells can be potentiated before transfer, to achieve a higher effectiveness. NK manipulation strategies that are under development include the genetic modification of NK cells, to obtain the enforced expression of genes coding for cytokines, such as IL-2, IL-15 and IL-12, for chemokine receptors for homing, or the overexpression of stimulatory receptors and the silencing of inhibitory ones

(Carlsten M and Childs RW, 2015). Chimeric antigen receptors (CAR), as previously performed in T cells, are currently developed for NK cells, to increase their recognition capability and functional activity (Carlsten M and Childs RW, 2015).

Recently a population with enhanced functional characteristics, named “memory” NK cells, has been described in $HCMV^+$ individuals (vide infra). The adoptive transfer of this population would be a promising strategy to be employed in tumor therapy. First attempts of infusion of these cells have been done, confirming their intriguing potential (Foley B et al., 2012a).

3. MEMORY NK CELLS

The immune system evolves during the lifetime shaped by a variety of factors; its functionality is the result of genetic factors, in combination with environmental events. Immunological profiling of homozygous twins highlight environment-dependent immune system differences, that increase with age, suggesting that a prevalent contribution is given by non-heritable factors (Brodin P et al., 2015). A greater functional response and the long-term protection after a previous exposure to pathogenic insult or other antigenic stimuli, is a recognized hallmark of the adaptive branch of the immune system, but a growing number of studies are highlighting that similar adaptation also occurs in innate cell populations, such as NK cells and monocyte-macrophages (Rölle A and Brodin P, 2016).

Indeed, it has been recently identified a distinct NK cell subset in humans, but also in mice and macaques, which shows peculiar characteristics: enhanced CD16-dependent effector functions, especially cytokine production, a distinct arrangement of surface marker and intracellular mediators, and long-term persistence *in vivo* (Holder KA et al., 2018).

3.1 HCMV-induced memory NK cells

Many viral infections confer long-term imprinting to the immune system, which results in enhanced protection to subsequent re-infections. A striking contribution to the shaping of the NK cell compartment is given by HCMV infection (Goodier MR et al., 2018; Rölle A and Brodin P, 2016).

As a result of complex strategies of immune evasion and co-evolution with the human species, HCMV is an exceptionally successful virus. Indeed, it is present in the majority of the human population: its prevalence ranges from 50 to 100%, depending on different socioeconomic and geographical contexts. HCMV infects a broad range of cell types, including epithelial cells of gland and mucosal tissue, macrophages, DC, hepatocytes and vascular endothelial cells. Similarly to other herpesviruses, after the primary infection the virus establishes a lifelong persistence inside myeloid cells in the BM, and switches his genetic program into a silent state, the so-called latency. HCMV can only occasionally reactivate, and contribute to viral transmission (Fields BN, 2013). In healthy individuals, primary

infection, as well as reactivation, is generally asymptomatic or sub-clinical; however in immunocompromised hosts, and rarely in immunocompetent subjects, it can cause severe disease (Riou R et al., 2017). Moreover, if the infection occurs during pregnancy, it can cause neurosensory damage to the foetus (Aronoff DM et al., 2017; Pereira L et al., 2017).

HCMV primary infection and subsequent reactivation raises a robust coordinate immune response that includes NK cells, as well as other components of cell-mediated and humoral immunity. NK cells are reported to be sufficient to control HCMV infection, even in subjects devoid of T cells, where it still associates to the expansion of a $NKG2C^+$ NK population (Kuijpers TW et al., 2008).

Many reports have documented a long-lasting modification of the NK cell compartment in healthy HCMV-infected individuals, whose mechanisms are still to be clarified (Rölle A and Brodin P, 2016).

A pioneering study showed an increase of $NKG2C^+$ NK cells in HCMV-seropositive individuals, with respect to seronegative ones. In parallel, a decrease of $NKG2A^+$ cells, its inhibitory counterpart, as well as of NKp30 and NKp46 activating receptors, was described. Preliminary longitudinal studies reported stability over time of these changes. Other receptors, such as NKG2D and KIR3DL1 showed no difference between serotypes (Gumá M et al., 2004). Further extensive characterization has identified other hallmarks of this population, such as the preferential expression of CD2, reduced expression of Siglec-7, CD161 and CD7 (Béziat V et al., 2012, 2013). Notably, and in accordance with the "memory" hypothesis, these cells are reported to be hyper-responsive to CD16 engagement *in vitro*, especially in terms of IFN-γ and TNF-α production (Hammer Q and Romagnani C, 2017; Rölle A and Brodin P, 2016).

Information on the *in vivo* dynamics of this population were provided by the privileged observation of the immune reconstitution in recipients of solid organ transplantation. The immunosuppressive treatment poorly affected NK cell compartment, so that it was possible to document the expansion of a $CD57^+NKG2C^{hi}$ NK cell subset, selectively in those patients in which HCMV reactivation occurred (Lopez-Vergès S et al., 2011; Foley B 2012a). The study of the reconstitution of the NK compartment in patients that underwent hematopoietic cell transplantation, and in which HCMV reactivation occurred, gave similar

results. Here, NK cells were the first to be reconstituted after transplantation, and the expansion of NKG2C$^+$ NK cells was reported, after viral reactivation. Moreover, these cells showed a more mature phenotype, with the increased expression of terminal differentiation markers, such as KIR and CD57, and the improved functional ability, in terms of IFN-γ production. Conversely, CD107 surface expression, considered a marker of cytotoxic granule mobilization, was not increased in NKG2C$^+$ cells compared to NKG2C$^-$ ones. These phenotypic and functional aspects were conserved up to 1 year after transplantation (Foley B et al., 2012b, 2014). Della Chiesa and colleagues reported similar results, in patients after umbilical cord blood transplantation. Indeed, the development of NK with an adaptive phenotype was restricted to HCMV$^+$ individuals (Della Chiesa M et al., 2012; Muccio L et al., 2018). Moreover the acquisition of NK cells with a fully memory phenotype needed a long timespan, up to 1 year (Muccio L et al., 2018).

The strict association of NKG2C$^+$ NK cells and HCMV seropositivity supports the idea that unknown HCMV infection-dependent mechanisms provide the necessary stimuli for the formation of this long living and highly functional population (Hammer Q and Romagnani C, 2017). A recent report of Romagnani's group contributed very interesting new data. They compared different allelic variants of the HCMV-encoded protein UL-40, whose peptide stabilizes HLA-E expression on the infected cell membrane. Some allelic variants from clinic isolates are preferentially recognized by CD94/NKG2C, and were shown to activate NKG2C$^+$ cell functional response. For peptides with suboptimal stimulation, co-stimulatory signals, as CD2 engagement, were necessary (Hammer Q et al., 2018). Moreover, they demonstrated that different UL-40 peptides lead to the formation of an NKG2C$^+$ cell population in NK cells isolated from HCMV seronegative individuals. These data suggest that viral factors can determine the shaping of the NKG2C$^+$ NK cell subset *in vivo*, and propose UL-40 protein as a possible driving factor (Hammer Q et al., 2018). This observation is in line with previous *in vitro* observations, where NKG2C blocking antibodies or knocking down HLA-E abrogate the expansion of NKG2C$^+$ NK cells (Rölle A et al., 2014).

Several reports have described a further expansion of the pre-existing NKG2C$^+$ NK cell pool in response to other viral infections. This association was verified for HBV, hantavirus, HIV, chikungunya and Epstein-Barr virus (Béziat V et al., 2012;

Björkström NK et al., 2011; Gumá M et al., 2006a, 2006b; Petitdemange C et al., 2011; Saghafian-Hedengren S et al., 2013). Altogether, these results suggest that the expansion of this peculiar NK cell subset in response to viral infections could be assimilated to a secondary response, while the first stimulus leading to the formation of this population could be represented by primary infection with HCMV.
This is a recognized feature of the immunological memory, hence this population has been called “memory” or “adaptive” NK cells (Rölle A and Brodin P, 2016). To explain the persistence of this population *in vivo*, the hypothesis that chronic viral infection and/or HMCV subclinical reactivation leads to the maintenance of memory NK cells has been proposed (Liu LL et al., 2015; Rölle A and Brodin P, 2016). Notably, and in accordance with the "memory" hypothesis, these cells are reported to be hyperresponsive to CD16 engagement *in vitro*, especially in terms of IFN-γ and TNF-α production (Hammer Q and Romagnani C, 2017; Rölle A and Brodin P, 2016).
Although NKG2C positivity is one of the first and more consistently described markers of memory NK cells, a NK cell population with similar phenotypic and functional features can be identified also in HCMV healthy individuals that are homozygous for a deletion of the NKG2C gene (5% of the general population), demonstrating that the establishment of memory NK cell population can be NKG2C-independent (Della Chiesa M et al., 2014; Liu LL et al., 2016). Since HCMV-expanded memory NK cells also display a skewed KIR repertoire, it was possible to identify these cells even in absence of the NKG2C marker. Thanks to the monitoring of KIR arrangement on the cell surface, it was possible to follow the expansion at the single cell level, and to identify the cell clones expanded in HCMV positive donors (Béziat V et al., 2013).
Among other markers, the lack of the FcεRIγ adapter is widely recognized as a characteristics of this memory NK population, so that the cells identified as $NKG2C^{+}$ or $FcεRIγ^{-}$ are mostly overlapping (Hwang I et al., 2012; Lee J et al., 2015; Schlums H et al., 2015; Zhang T et al., 2013). Interestingly the absence of the FcεRIγ chain is restricted to the $CD56^{dim}$ subset, that includes cells with mature phenotype (Hwang I et al., 2012), along with the previous cited papers that described the $NKG2C^{+}$ cells as terminally differentiated (Geary CD and Sun JC, 2017).

As reported by Hwang, the presence of a sizeable FcεRIγ$^-$ population was found only in 1/3 of the healthy individuals screened (Hwang I et al., 2012), and all of them had a previous exposition to the HCMV (Lee J et al., 2015; Zhou J et al., 2015). Notably, not all the individuals exposed to HCMV develop FcεRIγ$^-$ memory NK cells (Schlums H et al., 2015), suggesting the presence of other necessary factors linked to individual or virus variability.

Intracellular signalling molecules and functional signature of memory NK cells

Schlums and colleagues identified a distinct pattern of intracellular mediators associated with the NK adaptive phenotype, and mostly overlapping with the NKG2C$^+$ population: the lack of FcεRIγ, SYK tyrosine-kinase, Eat-2 and DAB2 intracellular adapters, and the Promyelocytic leukaemia zinc finger (PLZF) transcription factor (Lee J et al., 2015; Pérez-Quintero LA et al., 2014; Schlums H et al., 2015). CD3ζ expression, instead, was not affected (Hwang I et al., 2012). Notably, binding sites for PLZF were found on the FcεRIγ, SYK and EAT-2 promoters, suggesting a coordinate regulation of their expression in memory NK cells (Rölle A and Brodin P, 2016; Schlums H et al., 2015).

This peculiar asset of intracellular mediators may underlie the enhanced responsiveness to CD16 triggering (Schlums H et al., 2015). As mentioned before, CD16 can pair with either CD3ζ and FcεRIγ adapters, as homodimers or heterodimers. The number of activating ITAM domains differs in these adapters: FcεRIγ has one, while CD3ζ has three. If FcεRIγ is absent, CD16 can transduce only through the CD3ζ chain, that could result in a stronger downstream signal. This tuning of the intracellular mediators can be the reason why CD16 triggering is potentiated in memory NK cells (Hammer Q and Romagnani C, 2017; Lanier LL et al., 1991). Moreover CD16 expression is reduced in memory NK cells, suggesting that CD3ζ is a limiting factor for the expression/stability of CD16 on cell surface (Hwang I et al., 2012).

As mentioned before, memory NK cells, defined either as NKG2C$^+$ or FcεRIγ$^-$, have a peculiar functional profile that distinguishes them from the rest of “conventional” NK cells.

First, their natural cytotoxicity is impaired in response to classical target cells, such as K562 myelogenous leukemia cell line (Foley B et al., 2012b; Wu Z et al., 2013),

and comparable to conventional NK cells, against Raji lymphoblastoid cell line (Zhou J et al., 2015). Indeed, the lower expression of NKp46 and NKp30 FcεRIγ- and/or CD3ζ-coupled activating receptors may be responsible for the inferior responsiveness to some tumor targets (Hwang I et al., 2012; Schlums H et al., 2015; Zhang T et al., 2013). Moreover, memory NK cells are less responsive to IL-12 and IL-18 stimulation, in terms of IFN-γ production (Schlums H et al., 2015). The expression of the receptors for these two cytokines is under the control of PLZF transcription factor, whose levels are reduced in memory NK cells (Schlums H et al., 2015). All these data outlined the idea that "innate" responsivity, expressed as activation by proinflammatory cytokines or engagement of multiple activating receptors for natural recognition of altered cells, may be reduced in these cells, favouring a more selective, antibody-dependent recognition capability (Hammer Q and Romagnani C, 2017; Rölle A and Brodin P, 2016).

Indeed, several authors have reported a drastically enhanced memory NK activation in response to CD16 ligation, in terms of IFN-γ and TNF-α production, while it is still debated whether they also show an augmented release of cytotoxic granules (Costa-Garcia M et al., 2015; Hwang I et al., 2012; Lopez-Vergès S et al., 2011; Liu LL et al., 2016; Schlums H et al., 2015; Wu Z et al., 2013; Zhang T et al., 2013). In this regard, the requirement for the presence of a humoral response may represent a safeguard mechanism to avoid uncontrolled response of this hyperfunctional population (Rölle A and Brodin P, 2016).

The functional hyperresponsivity of memory NK cells is associated to epigenetic changes. The chromatin in the IFN-γ and TNF-α loci and distal regulatory elements was found globally demethylated in memory NK cells, thus providing a more open and accessible conformation, that favours the transcription of these genes (Luetke-Eversloh M et al., 2014; Schlums H et al., 2015). Interestingly, this hallmark was found only in seropositive individuals that had the expansion of $NKG2C^{hi}KIR^{+}$ NK cells. If no expansion occurred, $NKG2C^{+}$ cells were epigentically similar to the $NKG2C^{-}$ subset. Similar stable and heritable chromatin rearrangements are found in T_H1 and memory $CD8^{+}$ T cells, providing the hallmark of the IFN-γ producing cells (Luetke-Eversloh M et al., 2014). Reduced expression of PLZF can be a crucial factor In the creation and maintenance of these epigenetic modifications; indeed, it also interacts with enzymes for chromatin

modification (Mathew R et al., 2012; Schlums H et al., 2015).

Although their responsiveness to CD16 receptor engagement is considered a cardinal feature of this subset, other receptors can cooperate in triggering memory NK cell functional response. In fact, CD2 engagement has been recognized to have an important synergistic effect. This is an activating co-receptor expressed on NK cells that interacts with lymphocyte function-associated antigen 3 (LFA-3) on several cell types (Tangye SG et al., 2000). Notably, LFA-3 is upregulated on HCMV-infected fibroblasts (Rölle A and Brodin P, 2016). CD2 is expressed on NKG2C^{+} and NKG2C^{-} memory NK cells, and the co-engagement of CD16 and CD2 results in the improved production of TNF-α and IFN-γ (Liu LL et al., 2016). Moreover, NKG2C co-engagement displays a synergistic effect on CD16-induced cytokine release, linking the two main receptors that characterize memory NK population (Liu LL et al., 2016).

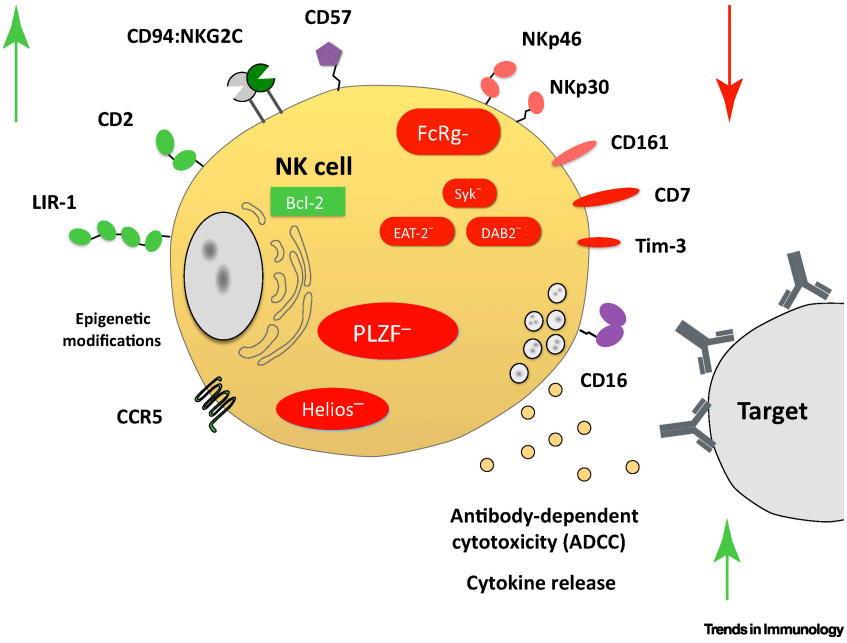

Figure 5. The molecular signature of memory NK cells

Memory NK cells differ from conventional NK cells for the expression of surface receptors, transcription factors, and key components of cellular signaling. Red indicates reduced or absent expression; green indicates increased expression or elevated function. All these features are not necessarily combined in a single cell (Rölle A and Brodin P, 2016).

3.2 Cytokine-induced human memory NK cells

NK cells that have been previously *in vitro* activated by cytokines alone display memory-like properties. This cytokine-driven memory has been observed in mice and humans, and may provide a powerful method for generating highly functional cells useful for immunotherapy (Hendricks DW et al., 2016).

The protocol to generate these cells includes *in vitro* culture with IL-12, IL-15 and IL-18, followed by an extended period of resting (Ni J et al., 2012; Romee R et al., 2012). These cultured memory NK cells displayed higher expression of CD94, NKG2A, NKG2C and CD69 (Romee R et al., 2012), showed enhanced proliferation, expression of the high-affinity IL-2 receptor (IL-2Rαβγ) (Leong JW et al., 2014; Romee R et al., 2016), and increased IFN-γ production upon stimulation with cytokines or with target cells, whereas cytotoxicity was not improved (Ni J et al., 2012; Romee R et al., 2012). These changes were maintained long-term and also inherited by daughter cells (Romee R et al., 2012). Notably, these cytokine-induced NK cells showed an improved response to acute myeloid leukaemia (AML) blasts *in vitro* and also when infused in mice bearing AML xenograft. In phase I trial, *in vitro* activated NK cell with IL-12, -15 and -18, led to the remission of AML patients (Romee R et al., 2016).

3.3 Memory NK cells in mice and non-human primates

In mice the first demonstration of an antigen-specific recall response by NK cells was reported in models lacking B and T cells. Here, for at least 4 weeks, a cytotoxic recall response to a specific hapten was conserved. Adoptive transfer of NK cells from a sensitized individual transferred hapten hypersensitivity to a non-exposed receiver (O'Leary JG et al., 2006). Other antigen-specific responses have been highlighted for multiple antigens, such as HIV gag pol env, and influenza virus M1 protein, imputable to liver, spleen and lung resident NK cell subsets (Paust S et al. 2010). Further studies documented NK-dependent antigen-specific responses to vaccinia virus and Mycobacterium tuberculosis (Gillard GO et al., 2011; Venkatasubramanian S et al., 2017).

During murine cytomegalovirus infection (MCMV), m175 viral glycoprotein is

recognized by Ly49H, an activating member of the Ly49 family, that contains activating and inhibitory partners. Upon ligand binding, the expansion of a $Ly49H^+$ NK cell population is triggered, and helps to control the primary infection and further reinfections (Beaulieu AM and Sun JC, 2016; Sun JC et al., 2009). Increasing evidence have revealed that an initial cytokine (type I IFNs, IL-12 and il-15) priming it is necessary, to promote aspecific activation and proliferation of NK cells (Orange JS and Biron CA, 1996; Venkatasubramanian S et al., 2017; Waldmann TA, 2015). Other co-stimulatory signals, such as DNAM-1 engagement, whose ligands are expressed on DC and monocytes after MCMV infection (Nabekura T et al., 2015), can be necessary for the complete development of this population (Nabekura T et al., 2014). Compared to conventional (non memory) NK cells, memory NK cells have a peculiar transcriptional profile; for example, it has been recently described that T-bet is necessary for their expansion and maintenance, while Eomes is not. Indeed, T-bet expression is up-regulated in MCMV infection, thanks to the effect of IL-12, that is abundant after viral challenge (Madera S et al., 2018). Adaptive NK cells show, moreover, enhanced IFN-γ production and degranulation in response to Ly49H and NK1.1 triggering *in vitro* (Sun JC et al., 2009).

Another interesting feature is that the pool of memory NK cells can be expanded just after the exposure to pro-inflammatory cytokines, even in absence of haptens or other ligand/receptor pairs. When murine splenic NK cells, shortly activated in vitro with IL-12, IL-15 and IL-18, are re-infused *in vivo*, they develop a stable population with enhanced IFN-γ production in response to re-exposure to the same cytokines or to receptor engagement. This enhanced functional response is maintained long term upon 12 weeks (Cooper MA et al., 2009).

In conclusion, in mice three paths lead to the development of memory NK cells with similar characteristics: hapten sensitization, microbe encounter, cytokine induction.

The existence of this population in mice brought to ask whether a similar subset exists in primates too. It has been demonstrated that rhesus macaque splenic NK cells can develop a prolonged response to simian immunodeficiency virus (SIV) or to human HIV antigens, up to 5 years post-vaccination. Indeed, liver and spleen NK cells isolated from SIV-infected macaques, selectively kill SIV antigen-pulsed

DC *in vitro* (Reeves RK et al. 2015). Another study described a memory NK cell population in spleen and peripheral blood of macaques, but without significant difference between SIV-infected and uninfected controls (Vargas-Inchaustegui DA et al., 2017).

AIM OF THE WORK

Recently identified memory NK cells display several interesting features that render them potentially interesting for exploitation in a therapeutical perspective. The aims of my experimental thesis were:

a) to evaluate the impact of environmental (HCMV seropositivity) and genetic (CD16 allelic variants) factors on the presence and amplitude of *in vivo* memory NK cell pool;

b) to assess the capability of therapeutic tumor-targeting mAb-opsonized tumor cells to promote CD16-dependent *in vitro* memory NK cell expansion;

c) to investigate phenotypic and functional characteristics of *ex vivo*-isolated and *in vitro*-expanded memory NK cell populations.

MATERIALS AND METHODS

Primary cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from anonymized healthy donor (HD) blood samples, obtained from the Transfusion Centre of the Policlinico Umberto I, Roma, and in accordance with the Declaration of Helsinki. Blood samples (5 ml) were collected in Lithium Heparin BD Vacutainer Tubes (BD Becton Dickinson, USA). PBMC were isolated by density-gradient centrifugation over Ficoll-Hypaque layer (Cederlane, Canada), according to the manufacturer's protocol.

Anti-HCMV IgG detection

HCMV seropositivity was assessed through the quantification of plasma anti-HCMV IgG levels, by using Anti-Cytomegalovirus (CMV) IgG ELISA Kit (Abcam) with the Immulite 2000 System (Siemens Healthineers), according to manufacturer's instructions. The analysis was performed by the Microbiology and Virology Unit, Policlinico Umberto I, under the supervision of Prof. O. Turriziani.

Cell lines

$CD20^+$ lymphoblastoid Raji B cell line, provided by Dr. F. D. Batista (Cancer Research UK, London), and K562 erithroleukemia, obtained from ATCC (USA), were cultured in RPMI 1640 (Euroclone, Italy), supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) Gibco (Thermo Fischer Scientific, USA) and 2 mM L-glutamine Sigma (Sigma-Aldrich, German) (complete medium), at 37° C, 5% CO_2 and 100% humidity. Cell lines were kept in culture for less than 2 consecutive months and routinely tested for positivity to mycoplasma presence.

NK cell expansion

Fifty-thousands PBMC per well were plated in round-bottomed 96 well cell culture plates (Sigma-Aldrich, Germany), in RPMI 1640 supplemented with 10% heat-inactivated FCS HyClone (GE Healthcare, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, Germany), 2 mM L-glutamine and 100

units/ml of human recombinant IL-2 (R&D USA). When specified, instead of IL-2, 10 ng/ml recombinant human IL-15 or 25 ng/ml recombinant human IL-21 (both from PeproTech, USA) were added to the culture medium. At day 2, 25,000 gamma-irradiated (3,000 rad) $CD20^+$ Raji cells were added, as such or after opsonization with saturating doses (1 µg/10^6 cells) of rituximab anti-CD20 therapeutic mAb (from Roche, Switzerland). Half-volume of the culture medium was replaced at day 4 and 7. Cultures were sacrificed at day 9 for phenotypic and functional analyses (**Figure 6)**. Cell numbers of the different lymphocyte subsets were obtained by combining absolute cell counts with cytofluorimetric data, per million of plated mononuclear cells. For functional analysis, 10 ng/ml of IL-15 were added to the IL-2-supplemented medium at day 7 of culture.

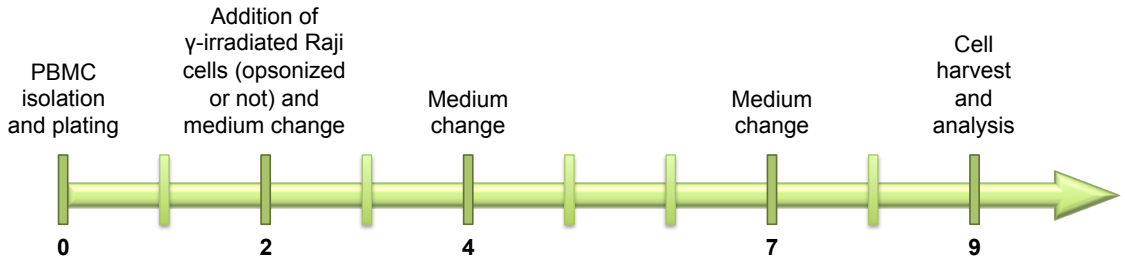

Figure 6. Memory NK cell *in vitro* expansion timeline

IL-2 (or IL-15 or IL-21) was present throughout the culture.

Immunostaining, flow cytometric analysis and gating strategy

Lymphocyte subsets were identified by surface staining with fluorochrome-conjugated monoclonal antibodies specific for surface markers, and intracellular staining with FITC-conjugated polyclonal anti-FcεRIγ subunit (see **Table 1** for antibody characteristics). To this end, cells were incubated with saturating concentrations of fluorochrome-conjugated monoclonal antibodies for 30 minutes at 4°C, and washed with washing buffer (PBS (Euroclone) supplemented with 2% FCS and 2 mM EDTA); subsequently, samples were fixed in 2% paraformaldehyde (Sigma) for 20 min at room temperature (RT), washed with washing buffer, permeabilized with 500 µl of washing buffer supplemented with 0.05 % Triton X-100 (BIO-RAD, USA) for 30 min at RT, washed again, and

incubated with anti-FcεRIγ subunit antibody for 30 min at 4°C; finally, samples were washed and resuspended in 100 µl of washing buffer, before analysis. Where necessary, twin samples were stained with isotype-matched control mAb, to set the marker for antigen positivity. For the detection of PLZF transcription factor, cell permeabilization and washings were performed with the eBioscience FoxP3/transcription factor staining buffer set (cat#: 00-5523-00, ThermoFisher Scientific), according to manufacturer's instruction.

The complete list of antibody used to detect surface markers and intracellular components is depicted in **Table 1**:

Antibodies	Clone	Company specification
anti-FcεRIγ subunit FITC	Polyclonal, rabbit	FCABS400F- Milli-Mark Merck
anti-CD56-APC	B159	555518- BD Biosciences
anti-CD56-APC-Vio770	REA 196	130-100-694- Miltenyi Biotec
anti-CD3 PerCP-Vio700	REA613	130-109-465- Miltenyi Biotec
anti-CD3 PerCP	SK7	347344- BD Biosciences
anti-CD16 PE-Vio770	REA423	130-106-706- Miltenyi Biotec
anti-CD16 PE	B73.1	347617- BD Biosciences
anti-CD16 FITC	3G8	555406- BD Biosciences
anti-CD16 FITC	MEM-154	MAB-1457F- Immunological Science
anti-NKp46 APC	REA808	130-112-122- Miltenyi Biotec
anti-NKG2C PE	134591	FAB138P- R&D Systems
anti-PD-1 PE	EH12.2H7	329906- BioLegend
anti-PD-L1 APC	29E.2A3	329708- BioLegend
anti-PLZF PE	Mags.21F7	12-9320-82- ThermoFisher Scientific
anti-IFN-γ APC	B27	554702- BD Biosciences
anti-CD107 PE	H4A3	555801- BD Biosciences
Mouse IgG PE	MOPC-21	555749- BD Pharmingen
Mouse IgG APC	MOPC-21	555751- BD Pharmingen
Mouse IgG FITC	MOPC-21	555748- BD Pharmingen
Mouse IgG PE	MOPC-173	400214- BioLegend
Mouse IgG APC	MPC-11	400322- BioLegend

Table 1. List of used antibodies

Samples were acquired with BD FACSCanto II and analyzed with FlowJo X.0.7 (TreeStar).

Memory NK cells were identified as $CD3^{-}CD56^{dim}Fc\varepsilon RI\gamma^{-}CD16^{+}$, as depicted in **Figure 7.** The threshold to consider a consistent memory population was set at 3% of total NK cells.

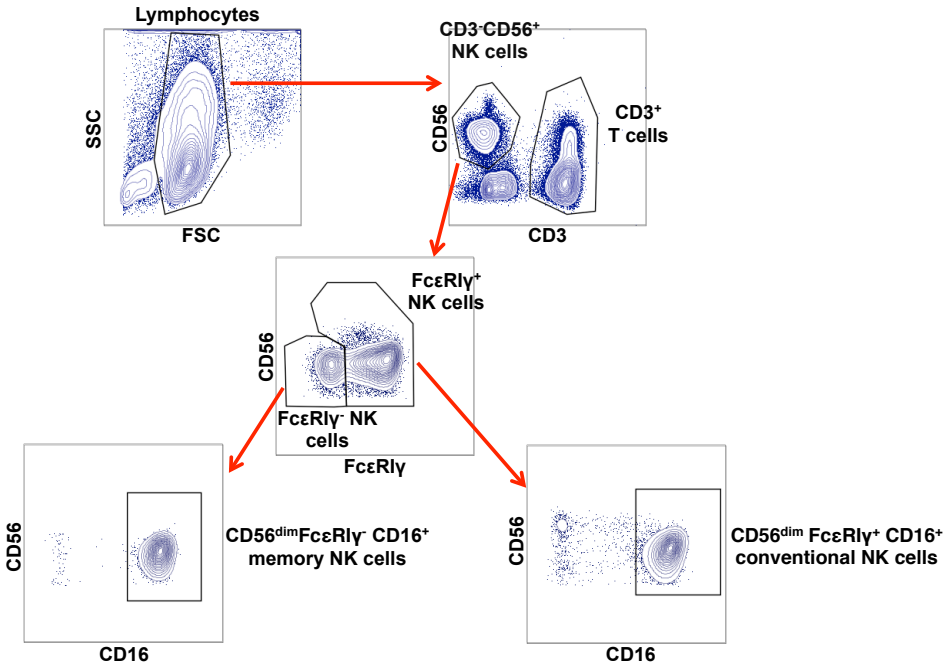

Figure 7. Gating strategy for the identification of memory and conventional NK cells by multiparameter flow cytometry

Cells were first gated on forward-scattered light (FSC), and on side-scattered light (SSC), indices of size and internal complexity. NK cells were selected for the expression of CD56 marker and the concomitant absence of CD3. Here, it was possible to distinguish cells based on the presence of FcεRIγ, among $CD16^{+}$ cells. Cells expressing FcεRIγ were defined conventional NK cells, while cells lacking FcεRIγ were the memory NK cells.

Characterization of CD16A polymorphism

CD16A (FcγRIIIa) allelic variants at position 158 (FcγRIIIa-158V/F) were predicted with a flow cytometry assay, as previously described (Böttcher S et al., 2005; Capuano C et al., 2017). This method exploits the capability of MEM-154 anti-CD16 mAb to recognize selectively the V158 variant. Thus, mean fluorescence intensity (MFI) of samples stained with MEM-154, normalized by MFI of staining

with 3G8 anti-CD16 mAb, whose affinity is unaffected by polymorphism at position 158, provides information on CD16 genotype (Böttcher S et al., 2005) (**Figure 8**). Accordingly, three samples for each donor are stained with APC-conjugated anti-CD56 and with either IgG-FITC, as negative control, or one of the two FITC-conjugated anti-CD16 mAb: 3G8 or MEM-154. The determination of each individual genotype is obtained by the ratio between the MFI of MEM-154 and 3G8 signals (calculated in the NK cell gate): F/F (ratio < 0.04), V/V (ratio > 0.62), and V/F (0.15 < ratio < 0.48) (Böttcher S et al., 2005). Capuano et al. previously validated this predictive method in our laboratory, by performing side-by-side gene sequencing analysis (Capuano C et al., 2017).

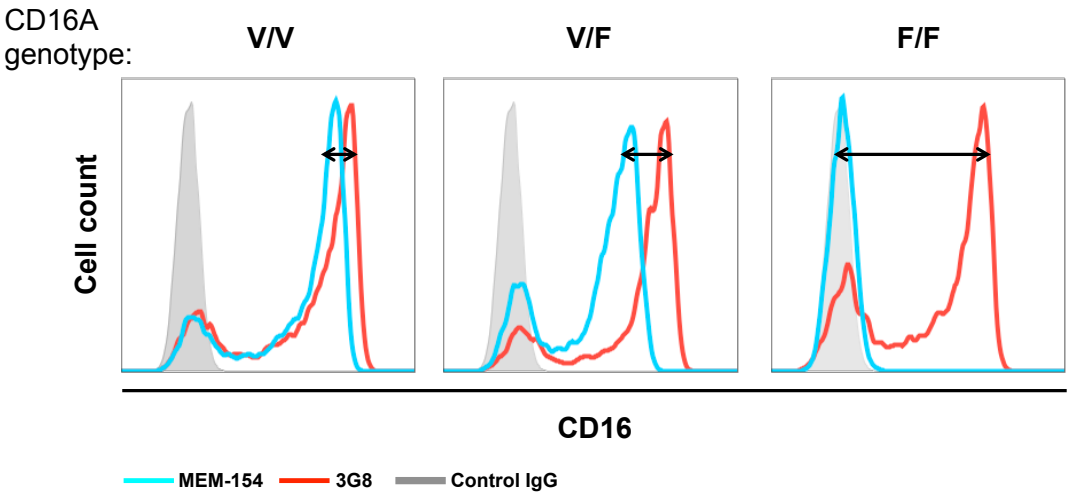

Figure 8. CD16 variants determination by flow cytometry

CD16 expression, detected with 3G8 or MEM-154 mAb, on NK cells in three donors bearing the three possible genotypes. MEM-154 (represented in blue) recognizes FcγRIIIa-158V with higher affinity than FcγRIIIa-158F; 3G8 binding is insensitive to allelic variants. MEM-154/3G8 MFI (mean fluorescence intensity) ratio allows to predict the three different genotypes.

Functional assays

Freshly isolated PBMC or cultured NK cell populations were stimulated with mAb-opsonized (or not) Raji, or with K562 target cells, in a E:T ratio of 2:1, in the presence of PE-conjugated anti-CD107a mAb, 50 µg/ml Monensin (Golgi-stop; cat #: M5273; Merck) and, after 1 hour, 10 µg/ml Brefeldin A (cat #: B7651; Merck).

Stimulation was stopped after 6 hours with 2 ml of cold PBS, and cells were washed at 1400 rpm for 10' at 4°C.

Samples were then stained for surface markers, fixed and permeabilized as described above, and stained with anti-IFN-γ APC and anti-FcεRIγ subunit FITC Ab.

Statistical analysis

Prism v.6 (GraphPad Software) and SPSS v24.0 (IBM Italia SpA) were used to perform statistical analysis. Differences between groups were calculated with two-tailed Mann-Whitney U test or Wilcoxon signed rank test, as appropriate; a p value <0.05 was considered significant.

RESULTS

1. *ex vivo* analysis of memory NK cells

1.1 Impact of HCMV seropositivity

We evaluated the presence of the memory NK population in a large cohort of 275 anonymous healthy donors (HD). They were identified as $CD3^{-}CD56^{dim}FcεRIγ^{-}$ $CD16^{+}$, according to the gating strategy depicted in **Figure 7**. We found that a discrete population, estimated as more of 3% of total NK cells, was present in 52% of HD (data not shown).

Since HCMV infection has been proposed as a required event for memory NK cell development in humans (Rölle A and Brodin P, 2016; Hammer Q and Romagnani C, 2017), we analyzed virus-specific IgG titers in a sub-cohort of 169 healthy donors, in which 74.6% (123) were found to be $HCMV^{+}$ (**Figure 9A**, upper panel). The presence of a sizeable (exceeding the set 3% threshold) population of memory NK cells was almost completely restricted to seropositive individuals (**9B**, left panel). At variance, $CD3^{-}$ $CD56^{+}$ NK cell percentage among circulating lymphocytes was not significatively different between HCMV seropositive and seronegative individuals (**9B**, right panel).

We found that 53.7% of $HCMV^{+}$ donors had a memory NK cell population of an intermediate size (3-20% of total NK cells), 13% of them had a large population (>20% of NK cells), and 33.33% of $HCMV^{+}$ HD did not show a sizeable population of memory NK cells (<3% of total NK cells) (**9A**, lower panel).

Despite the tight connection between HCMV infection and memory NK cell development, we observed no correlation between the percentage of memory NK cells and the plasma titer of HCMV-specific IgG (Spearman r= 0.04135, p= ns) (**9C**). This lack of correlation stood true also when we took into account only those donors (13%) with an expanded memory NK cell population (more than 20% of total NK cells - Spearman r= -0.06181, data not shown)

These results show, on a large cohort of healthy individuals, the great quantitative heterogeneity of the memory NK cell subset. They confirm data from the literature

that identify HCMV seropositivity as a pre-requisite for the *in vivo* presence of memory NK cell pool (Rölle A and Brodin P, 2016; Hammer Q and Romagnani C, 2017), imply that other factors are involved in the establishment and amplitude of this specific subset, and reveal that individual variations of the anti-HCMV antibody response do not impact the size of memory NK cell pool.

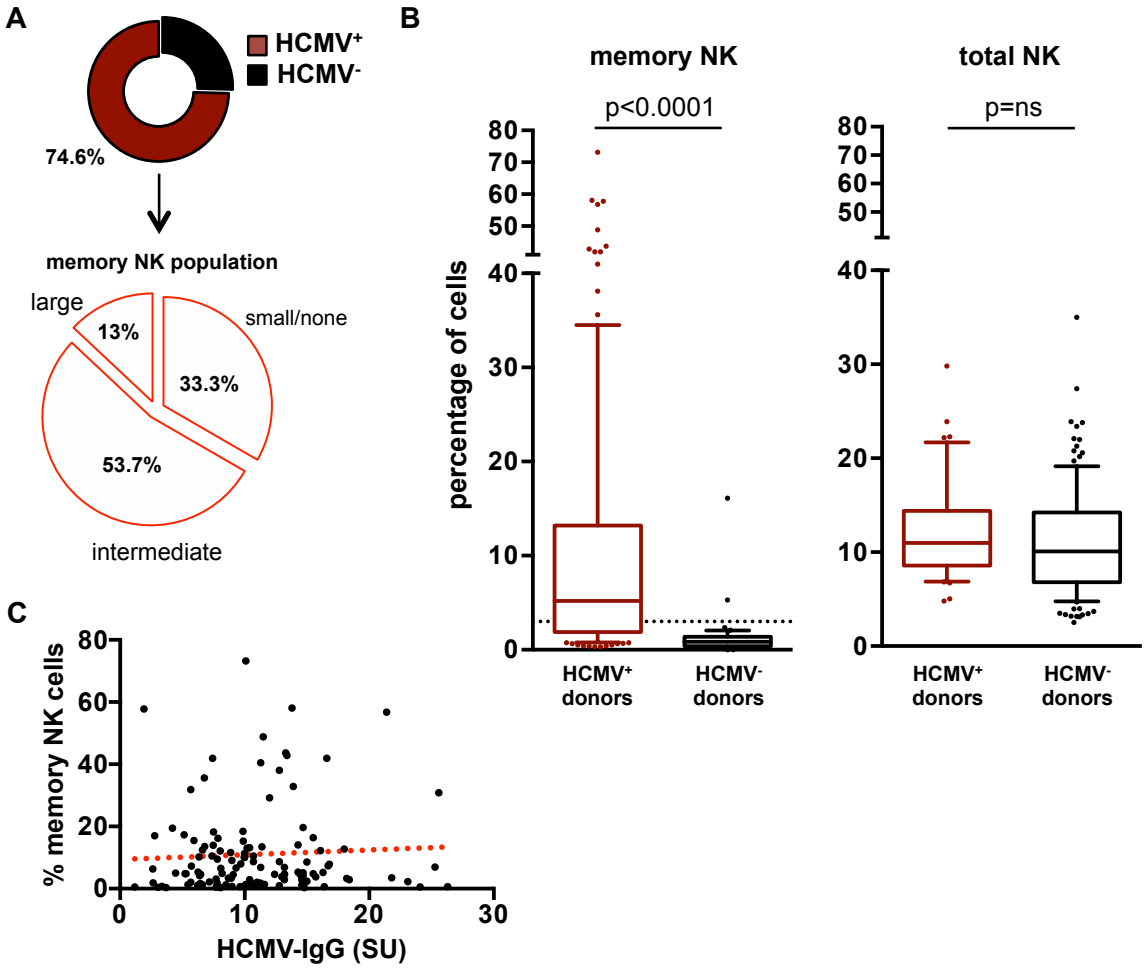

Figure 9. *Ex vivo* quantification of memory NK cells and HCMV serostatus

A. Upper panel: HCMV serostatus in a cohort of 169 healthy donors (HD); lower panel: HCMV$^+$ individuals were divided according to the size of the memory NK cell population (CD3$^-$ CD56^{dim}FcεRIγ$^-$CD16$^+$): no sizeable (less than 3% of NK cells), intermediate size (3-20%) or large size (>20%) population. **B.** Percentages of memory (left panel) and total (CD3$^-$CD56$^+$) (right panel) NK cells in HCMV$^+$ (red) and HCMV$^-$ (black) HD. The dotted line represents the 3% threshold. Box and whiskers graphs represent the median of the distribution and the 10-90 percentiles. Outliers are shown as single dots. P values were assessed by Mann-Whitney non-parametric test**. C**. Lack of correlation between the percentage of memory NK cells and the titer of plasma anti-HCMV IgG (SU, Standard Units), as estimated with Spearman correlation analysis.

1.2. Impact of CD16 polymorphisms

It has been proposed that memory NK cell maintenance *in vivo* could be supported by CD16 stimulation, upon encounter with Ab-opsonized infected cells during HCMV subclinical reactivation in healthy immunocompetent hosts (Lee J et al., 2015; Schlums H et al., 2015; Zhang T et al., 2013). To evaluate whether CD16 polymorphism at position 158, that affects the affinity of CD16 for IgG Fc portion ligand, could impact the presence and the abundance of the memory population *in vivo*, 101 $HCMV^+$ HD were stratified by their CD16 allotype: V/V (high affinity), V/F (intermediate affinity) or F/F (low affinity), as predicted by immunostaining with MEM-154 allele-sensitive mAb (**Figure 10A**, pie graph) (Böttcher S et al., 2005; Capuano C et al., 2017).

The fraction of individuals bearing a sizeable (>3% of total NK cells) memory NK cell population was comparable across genotypes (**10A**, bar graphs).

However, since CD16 is a driver of NK cell proliferation (Lee HR et al., 2017), receptor allotypes could be relevant in determining the size of memory NK cell pool *in vivo*; we thus compared the distribution of the percentage of memory NK cells in the three genotypes (**10B**). Our analysis showed no significant differences in the percentage of memory NK cells, and of total NK cells as well, among individuals belonging to the three allotypes; however, memory NK cells were slightly less abundant in VV donors.

Taken all together, our data show that CD16 affinity for IgG, as determined by allele variants at position 158, does not significatively affect either the development or size of the memory NK cell pool *in vivo*.

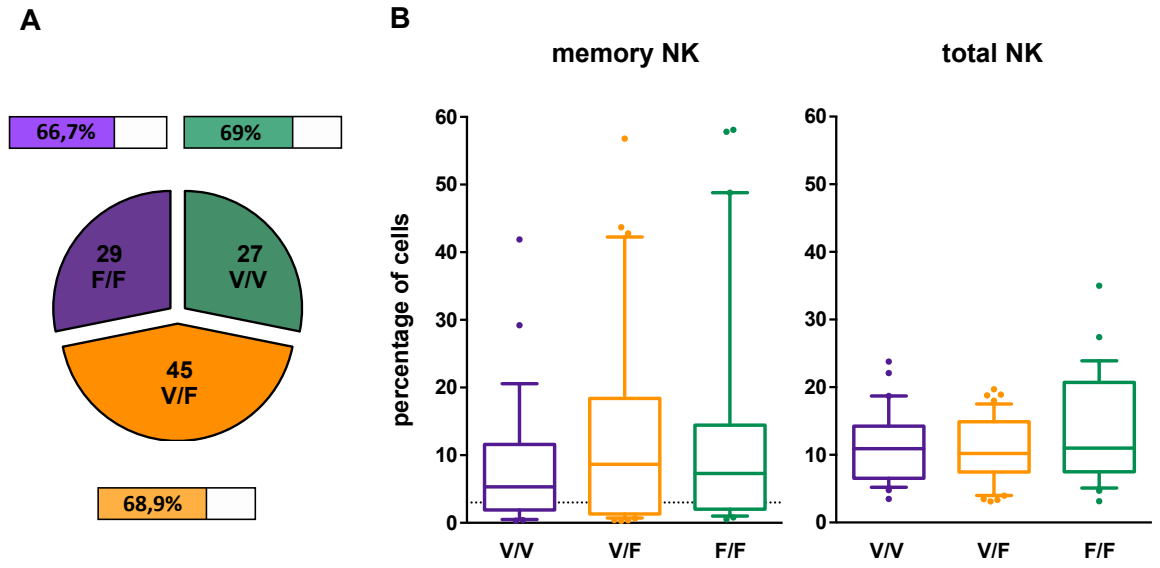

Figure 10. Relationship between *ex vivo* memory NK cell abundance and CD16 polymorphisms

A. CD16 gene variants at position 158 were assessed in a cohort of 101 HCMV$^+$ healthy donors (HD). HD numbers bearing each genotype (pie graph), and percentage of individuals showing a sizeable (>3% of total NK cells) memory NK cell population, for each genotype (bars). **B.** Percentage of memory (CD3$^-$CD56^{dim}FcεRIγ$^-$CD16$^+$, left) and total (CD3$^-$CD56$^+$, right) NK cells in individuals carrying the three CD16 genotypes. Box and whiskers graphs represent the median of the distribution and the 10-90 percentiles. Outliers are shown as single dots. P values were assessed by Mann-Whitney non-parametric test. No pairwise comparison reached the significance threshold.

1.3. Analysis of CD16 expression

It has been reported that memory NK cells express CD16 receptor with a lower intensity than conventional NK cells (Hwang I et al., 2012). We confirmed that CD16 expression level is significatively lower in memory NK cells, as compared to "conventional" ones (**Figure 11A**, upper panel), in a large cohort of 104 HCMV$^+$ HD, and as shown in a representative donor (**11A**, lower panel). Here we show that the lower expression of CD16 on memory NK cells, as compared to "conventional" (CD3$^-$CD56^{dim}FcεRIγ$^-$CD16$^+$) is consistently observed across CD16 genotypes (**11B**). This occurs despite the fact that both memory and conventional NK cells of high affinity (V/V homozygous) donors bear lower levels of CD16, when compared with the respective populations of the low affinity (F/F

homozygous) individuals (**11B**).

Collectively taken, these data confirm previous evidence in the literature that FcεRIγ^{-} memory NK cells express CD16 receptor with a lower intensity, and indicate that this phenotypic characteristics is maintained notwithstanding genetic factors that affect receptor surface levels.

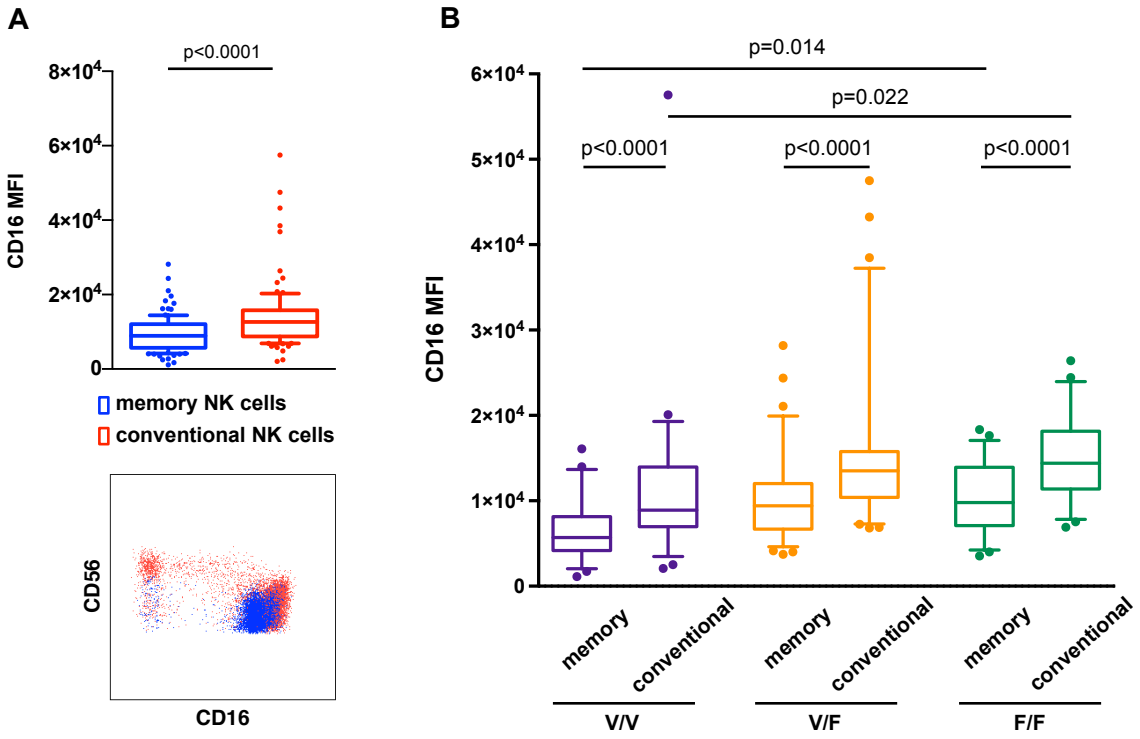

Figure 11. *Ex vivo* analysis of CD16 intensity on memory NK cells

A. CD16 intensity (expressed as mean fluorescence intensity, MFI) on memory (CD3^{-} CD56^{dim}FcεRIγ^{-}CD16^{+}, blue) and conventional (CD3^{-}CD56^{dim}FcεRIγ^{+}CD16^{+}, red) NK cells in a cohort of 104 HCMV^{+} individuals (upper panel) and in a representative donor (lower panel). **B**. CD16 intensity (expressed as MFI) on memory and conventional NK cells in individuals of the three CD16 genotypes (same cohort of Figure 10). Box and whiskers graphs represent the median of the distribution and the 10-90 percentiles. Outliers are shown as single dots. P values were assessed by Mann-Whitney and Wilcoxon tests, as appropriate.

1.4. Memory NK cell distinctive surface markers and intracellular mediators

We then analyzed the expression of surface and intracellular markers that have been previously reported to have a distinctive expression on memory NK cells

(Schlums H et al., 2015; Hwang I et al., 2012; Lee J et al., 2015).

Indeed, memory NK cells were enriched for the presence of NKG2C$^+$ cells, while the percentage of NKp46 cells, and the levels of PLZF transcription factor, were lower on the memory NK cell subset, than their conventional counterpart (**Figure 12**).

We then focused our attention on the expression of the immunological checkpoint PD-1 and its ligand PD-L1, as this receptor/ligand system may regulate memory NK cell interaction with tumor targets and with other immune system components (Bardhan K et al., 2016). PD-1 checkpoint receptor was reported to be expressed on a limited NK cell subset, in only a fraction of healthy individuals (Della Chiesa M et al., 2016; Pesce S et al., 2017). PD-1 was expressed at detectable levels on 13/17 donors in our cohort, albeit at low levels and without any significative difference between memory and conventional NK cells (**Figure 12A**). Interestingly, here we first show that human NK cells express PD-L1, and that the percentage of PD-L1$^+$ cells is significatively lower in memory NK cells than in their conventional counterpart (**12A**).

These results confirm and extend the characterization of the distinctive phenotypic profile of freshly isolated human memory NK cells.

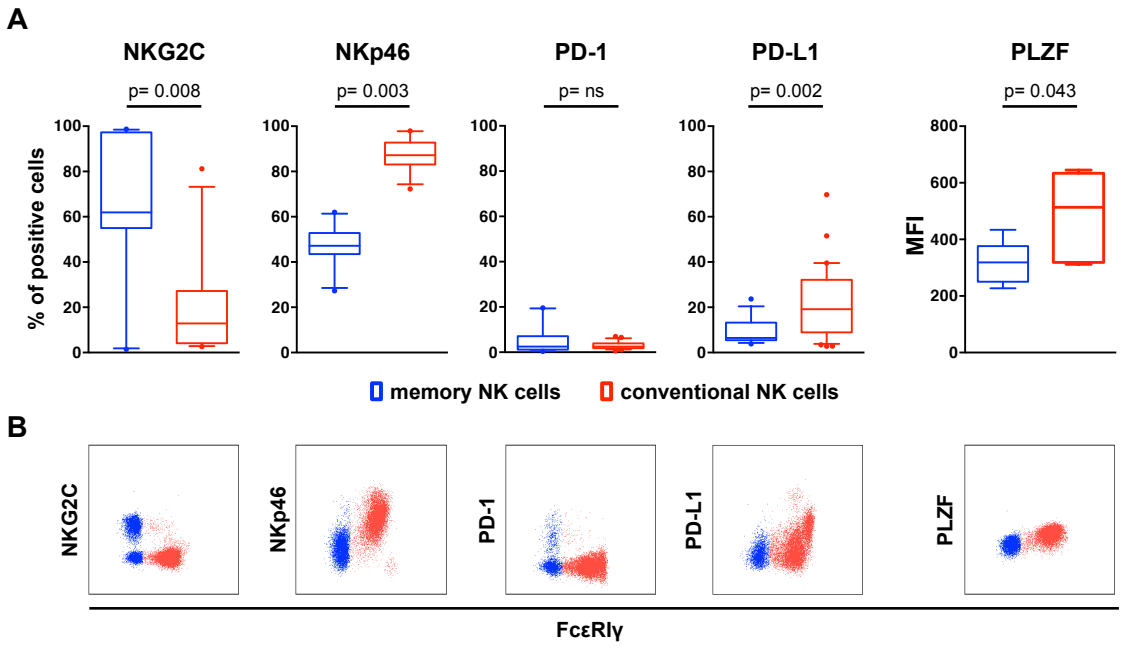

Figure 12. Distinctive arrangement of memory NK cell markers

A. Expression of NKG2C, NKp46, PD-1, PD-L1 and PLZF on memory ($CD3^-CD56^{dim}Fc\varepsilon RI\gamma^-CD16^+$) and conventional ($CD3^-CD56^{dim}Fc\varepsilon RI\gamma^+CD16^+$) NK cells. 11 HD were analyzed for NKG2C and NKp46 expression, 6 for PZLF, 17 for PD-L1 and PD-1. Box and whiskers graphs represent the median of the distribution and the 10-90 percentiles. Outliers are shown as single dots. P values were assessed by Wilcoxon test for paired samples. **B.** NKG2C, NKp46, PD-1, PD-L1 and PLZF expression are shown for representative donors.

2. *in vitro* expansion of memory NK cells

2.1 Characterization of the ability of tumor-targeting mAb-opsonized lymphoblastoid cells to promote memory NK cell proliferation

It has been reported that memory NK cells can proliferate *in vitro*, upon stimulation with infected target cells opsonized with antiviral antibodies (Zhang T et al., 2013; Lee J et al., 2015).

Therefore, we asked if tumor cells opsonized with therapeutic tumor-targeting mAb could drive the expansion of this NK cell subset. To investigate this, we set up an expansion protocol where memory NK cell-containing PBMC from healthy donors were co-cultured for 9 days with γ-irradiated $CD20^+$ B cell lymphoblastoid Raji target cells, opsonized or not with RTX anti-CD20 therapeutic mAb, in the

presence of IL-2. At day 9, cells were harvested and analyzed by immunostaining and flow cytometry. The experimental protocol is summarized in **Figure 6**.

Our results clearly show that while conventional ($CD3^-CD56^{dim}Fc\varepsilon RI\gamma^+CD16^+$, red) NK cells comparably proliferated, either in the presence or in the absence of RTX, memory ($CD3^-CD56^{dim}Fc\varepsilon RI\gamma^-CD16^+$, blue) NK cells proliferate only in the presence of RTX-opsonized Raji (**Figure 13**). These results suggest that memory NK cell proliferation is selectively driven by stimuli triggered through CD16 ligation. These data demonstrate that *in vitro* exposure to RTX-opsonized leukemia cells selectively induces memory NK cell proliferation. They also show that memory NK cell proliferation displays a large donor-to-donor variation. The magnitude of

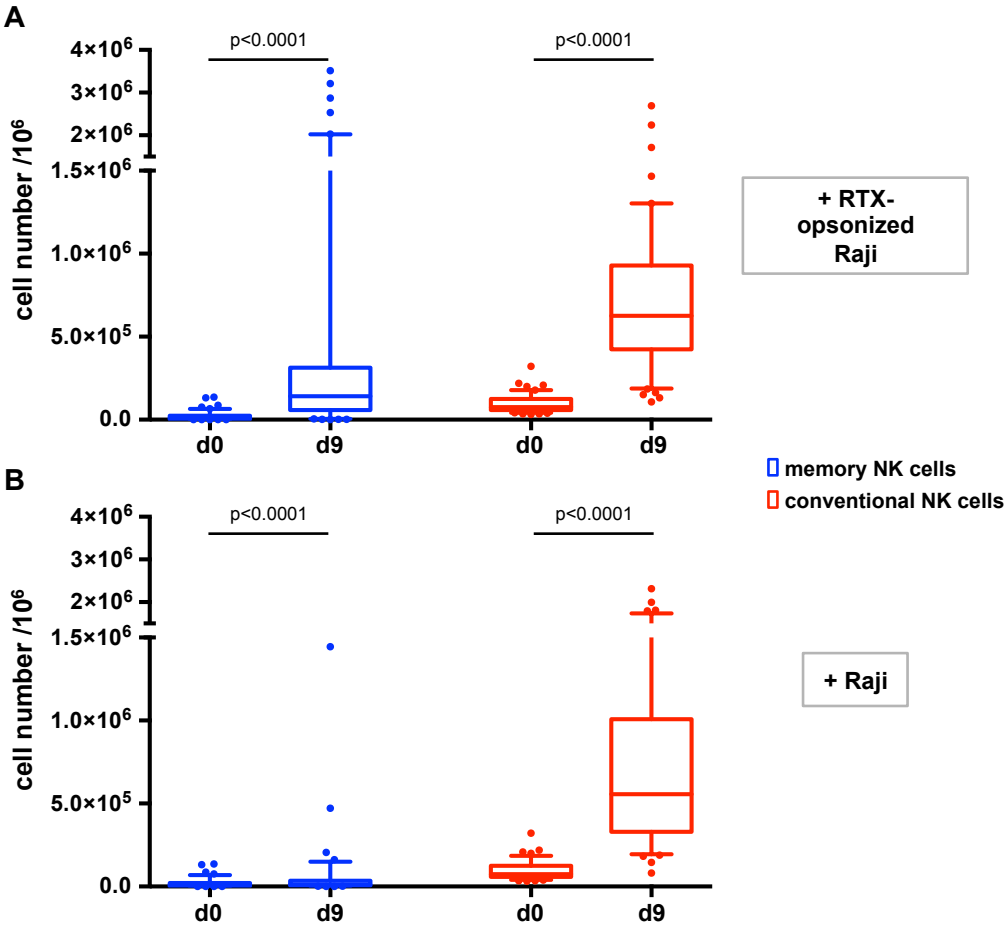

Figure 13. *In vitro* memory NK cell expansion with RTX-opsonized Raji

Proliferation of memory NK cells (blue) and conventional NK cells (red), in the presence of IL-2 and Raji leukemia cell line, opsonized (**A**) or not (**B**) with saturating doses of RTX, in a cohort of 46 $HCMV^+$ HD. Box and whiskers graphs represent the median of the distribution and the 10-90 percentiles. Outliers are shown as single dots. P values were assessed by Wilcoxon test.

proliferation did not correlate with the size of the subset in the starting population (day 0 PBMC), as shown in **Figure 14A.**

We then asked whether *in vitro* exposure to tumor-targeting mAb-opsonized lymphoblastoid cells could induce the expansion of memory NK cells from peripheral blood populations that lacked a sizeable pool (<3% of total NK cells). Interestingly, a significant *in vitro* expansion could be observed, but only in $HCMV^+$ donors, while no proliferation was observed when PBMC derived from $HMCV^-$ individuals (**Figure 14B**). Thus, despite the memory NK cell population was similar for small size (3% of NK cells) in both seropositive and seronegative individuals, the previous history of HCMV infection determined the ability of these cells to expand *in vitro,* in our experimental conditions.

Additionally, RTX-dependent memory NK cell *in vitro* expansion did not significantly differ among the three CD16 genotypes (**Figure 15**), suggesting that the strength of Fc ligation does not affect population expansion ability, under our experimental conditions.

Our standard model of memory NK cell expansion includes the presence of IL-2 to sustain cell proliferation. We asked whether IL-15 and IL-21 cytokines, that have

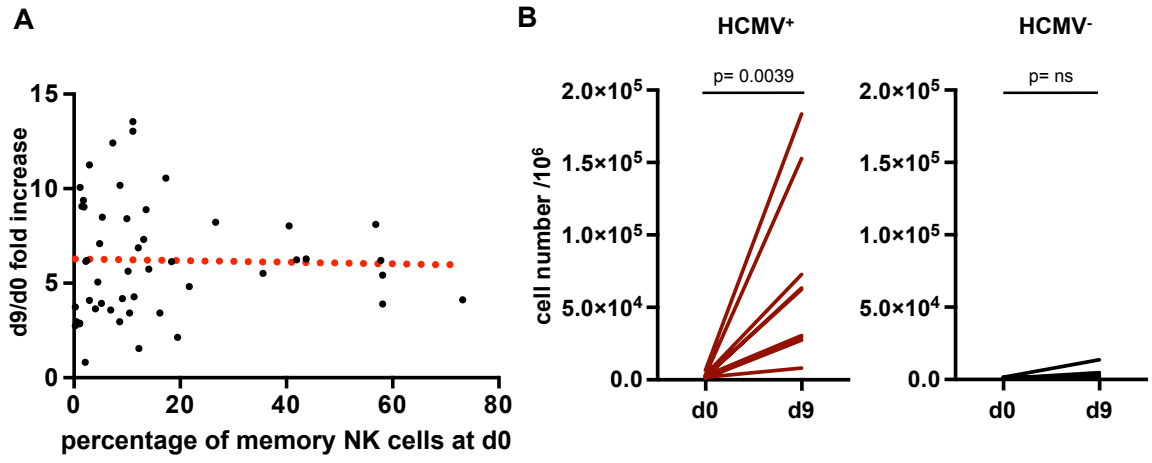

Figure 14. *in vitro* memory NK cell expansion with RTX- opsonized Raji

A. Absence of correlation between the ratio of expansion (ratio of the absolute number of cell harvested at day 9 on cell plated at day 0) and the size of memory NK cell pool at day 0. Correlation was assessed by Spearman test. **B.** Expansion of memory NK cells in HCMV seropositive (9 HD, left) and seronegative (6 HD, right) PBMC containing less than 3% of memory NK cells, in the presence of RTX-opsonized Raji. P values were assessed by Wilcoxon test.

all been demonstrated to act on NK cells (Pillet AH et al., 2009; Parrish-Novak J et al., 2002), and whose receptors belong to same family and share part of the signal transduction pathway, could have the same role in promoting memory NK cell proliferation.

We then set up co-cultures of PBMC from memory NK cell-containing HCMV$^+$ individuals with RTX-opsonized or control Raji cells, in the presence of IL-2, IL-15 or IL-21. We observed that IL-15 was comparable to IL-2 in promoting RTX-dependent memory NK cell proliferation, while IL-21 was less efficient. However, IL-15 supported RTX-independent proliferation of memory NK cells significatively better than IL-2 and IL-21 (**Figure 16A**). Notably, the three cytokines differently impacted on conventional NK cell proliferation. In fact, IL-21 was superior than IL-2 and IL-15 in supporting conventional NK cell proliferation, in the presence of either RTX-opsonized or control Raji cells; while IL-15 did not significantly differ from the standard condition (IL-2) (**Figure 16B**).

Collectively taken, although obtained in a limited number of individuals, these results suggest that memory NK cells have different sensitivity to the cytokines of the IL-2 family than conventional NK cells, in our experimental system.

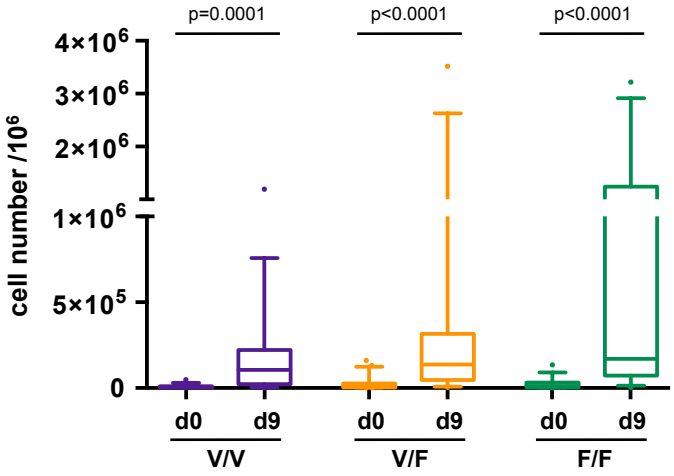

Figure 15. CD16 genotype does not affect the *in vitro* expansion of memory NK cells

Expansion of memory NK cells in co-culture with RTX-opsonized target cells, stratified by CD16 genotype. Number of HD per genotype: 14 V/V; 18 V/F; 18 F/F. Box and whiskers graphs represent the median of the distribution and the 10-90 percentiles. Outliers are shown as single dots. P values were assessed by Wilcoxon test for paired samples. The statistical comparison among different genotypes was performed with Mann-Whitney test, and gave no significant differences.

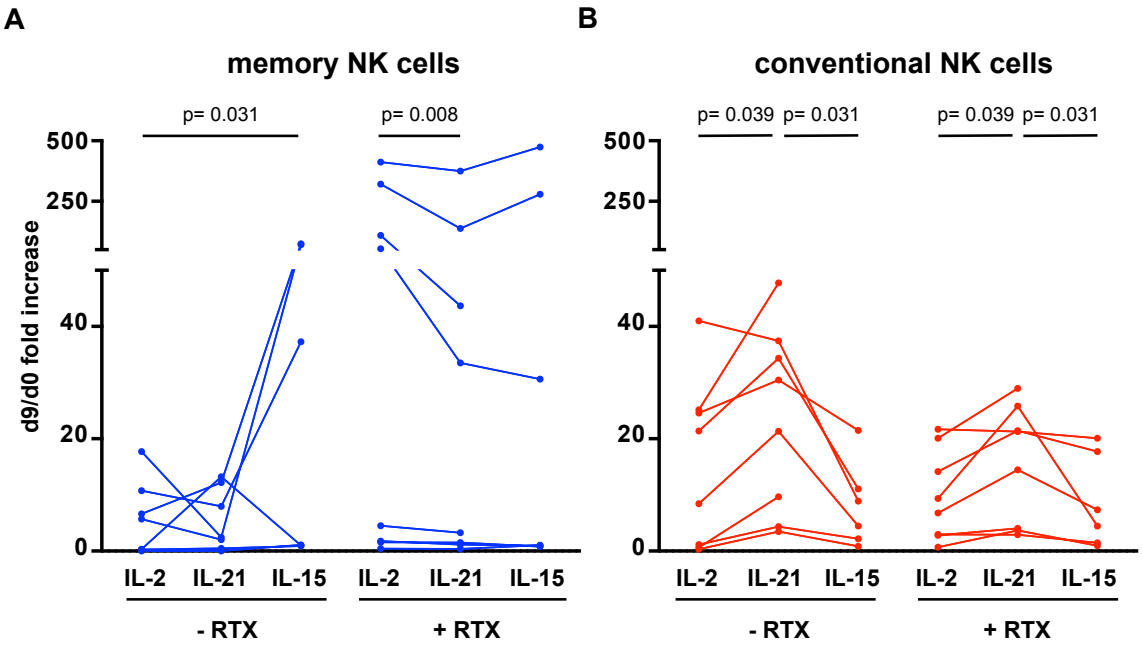

Figure 16. Comparison *of* memory NK cell expansion in the presence of IL-2, IL-21 or IL-15
Memory (**A**) and conventional (**B**) NK cell expansion with RTX-opsonized or not opsonized Raji cells, in the presence of IL-2, IL-21 or IL-15. The contribution of the three different cytokines to the cell proliferation (ratio of absolute number of cells at day 9/day 0). p values were assessed with Wilcoxon test for paired samples.

2.2 Phenotypic profile of in vitro cultured memory NK cells

To assess whether our *in vitro* culture protocol may affect the phenotype of memory NK cells, we analyzed, on *in vitro* expanded memory NK cells, the same array of markers evaluated on the fresh population (see **Figure 12**).

Our results show that, despite the expression levels of those markers were modulated upon *in vitro* culture, the distinctive pattern of expression, as compared to conventional NK cells, was maintained**.** In fact, *in vitro* cultured memory NK cells expressed higher levels of NKG2C, and lower levels of NKp46, CD16 and PLZF transcription factor (**Figure 17).**

Interestingly, PD-L1 stably remained on a low percentage of memory NK cells, but after the *in vitro* culture its expression was drastically lost on conventional NK cells (**Figure 17**).

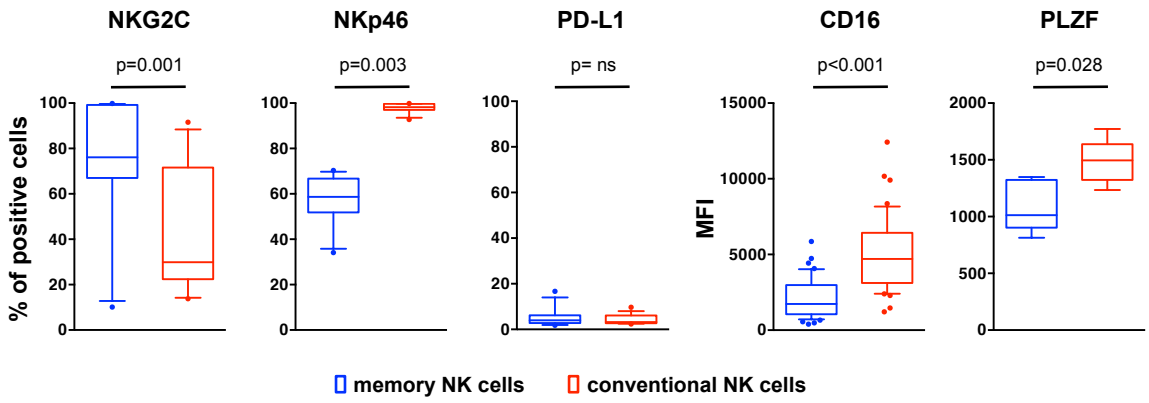

Figure 17. The distinctive arrangement of memory NK cell markers is maintained after *in vitro* culture

Expression of NKG2C, NKp46, PLZF, PD-L1 and CD16 on memory ($CD3^{-}CD56^{dim}Fc\varepsilon RI\gamma^{-}CD16^{+}$, blue) and conventional ($CD3^{-}CD56^{dim}Fc\varepsilon RI\gamma^{+}CD16^{+}$, red) NK cells. 11 HD were analyzed for NKG2C and NKp46 expression, 6 for PZLF, 17 for PD-L1 and 46 for CD16. Box and whiskers graphs represent the median of the distribution and the 10-90 percentiles. Outliers are shown as single dots. P values were assessed by Wilcoxon test.

2.3. Functional profile of fresh and *in vitro* cultured memory NK cells

The ability of memory NK cells to produce IFN-γ more efficiently than conventional ones in response to CD16 stimulation has been described by several authors (Hwang I et al., 2012; Zhang T et al., 2013; Lee J et al., 2015; Liu LL et al., 2016). We quantified the percentage of multifunctional memory NK cells, in both freshly isolated and *in vitro* cultured populations, in response to RTX-opsonized Raji cells. As shown in **Figure 18A**, fresh memory NK cells displayed a significatively higher percentage of cells capable of simultaneous IFN-γ production and degranulation, evaluated as externalization of CD107a cytotoxic granule marker, as compared to their respective conventional counterpart. Conversely, stimulation with K562 erythroleukemia cells, a paradigmatic natural cytotoxicity target, resulted in a lower response of memory NK cells, as previously noted (Foley B et al., 2012b; Wu Z et al., 2013). Non-opsonized Raji displayed a poor capability to induce multifunctional response of either memory and conventional freshly isolated populations. *In vitro* culture induces a general increase in responsiveness, for both memory and conventional NK cell subsets, probably attributable to the presence of cytokines (IL-2 throughout the 9 days of culture, and IL-15, specifically added for the last two

days of culture, in this experimental setup) (**18B**). Neverthess, the enhanced response to CD16 stimulation, although tendential, and the lower responsivity to K562 target, are conserved features of memory NK cells.

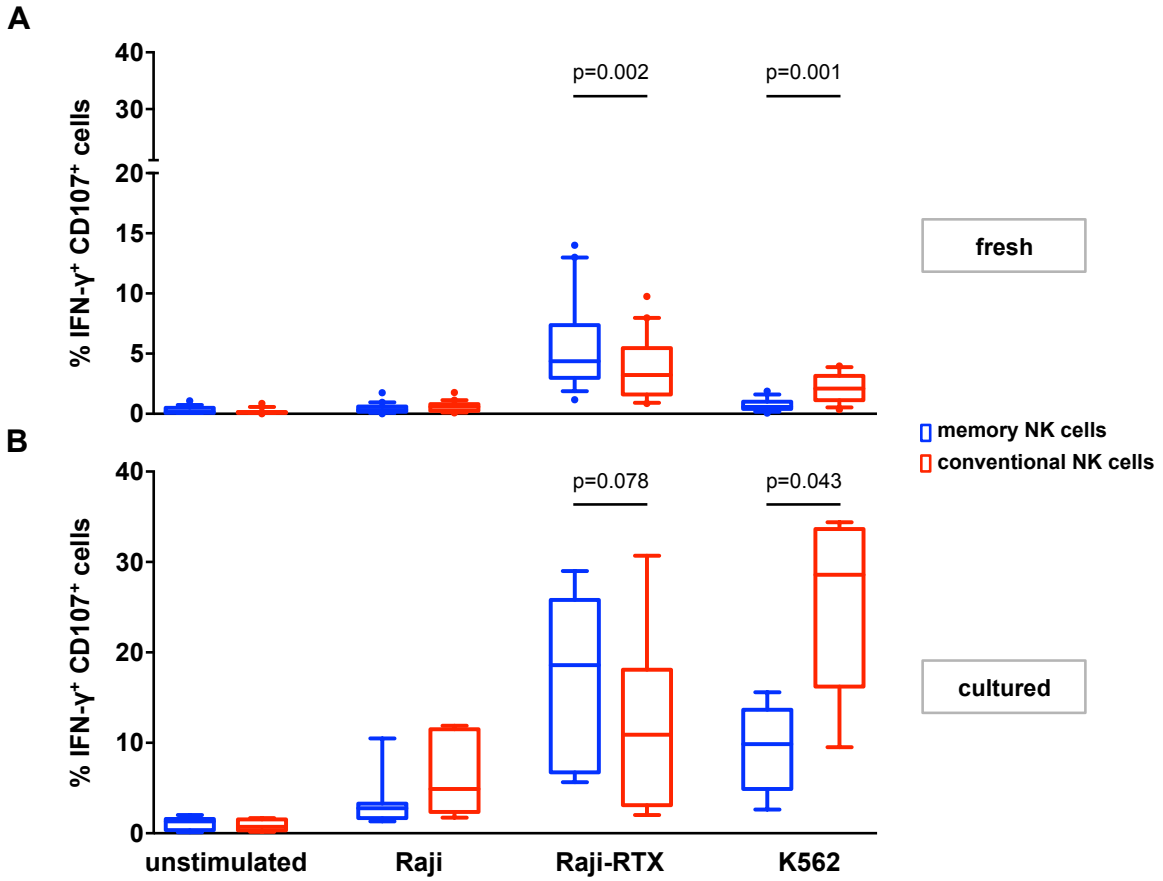

Figure 18. Multifunctional response of memory and conventional NK cells

Stimulation of fresh (**A**) and cultured (**B**) memory (blue) and conventional (red) NK cells (19 and 7 HD, respectively) with Raji, RTX-opsonized Raji or K562 cells. The percentage of IFN-γ-producing and surface CD107-expressing cells is reported. Box and whiskers graphs represent the median of the distribution and the 10-90 percentiles. Outliers are shown as single dots. The statistical significance was assessed with Wilcoxon test.

DISCUSSION

NK cells are important players of the immune response against cancer, crucial participant in tumor surveillance, and may also be exploited as an attractive tool for cancer immunotherapy strategies (Fang F et al., 2017; Gajewski TF et al., 2013).

Recently, the great heterogeneity and plasticity of this lymphocyte population is increasingly appreciated. In particular, the identification of a NK cell subset with memory-like characteristics (e.g. long-living and highly functional) rose the attention and the interest of the scientific community (Geary CD and Sun JC, 2017; Hammer Q and Romagnani C, 2017).

Our study provides an additional characterization of human fresh memory NK cells, and addresses the role of genetic and non-genetic host factors in affecting their development and abundance.

Our data confirm and extend, on a large cohort of healthy donors, that HCMV previous infection is a pre-requisite condition for the appearance of peripheral blood memory NK cells (Lee J et al., 2015; Zhou J et al., 2015). In fact, the presence of a sizeable (>3% of all NK cells) population of memory NK cells, defined as $CD3^{-}CD56^{dim}Fc\varepsilon RI\gamma^{-}CD16^{+}$, was almost completely restricted to HCMV-seropositive individuals. The systemic anti-viral immune response results in the presence of proinflammatory cytokines, virus antigens and HCMV-specific antibody-opsonized infected cells, during virus reactivation episodes. It has been proposed that a combination of these factors may lead to the development of the memory NK cell subset (Geary CD and Sun JC, 2017; Hammer Q and Romagnani C, 2017). Nevertheless, HCMV infection seems not a sufficient factor, as memory NK cells are found expanded (>3% of NK cells) in only a fraction (66%) of HCMV seropositive individuals.

Our analysis showed a large variability in the size of the *in vivo* memory NK cell pool, which may be related to inter-individual differences in the host immune response. We could assess that the intensity of the anti-viral response, evaluated as the plasma titer of HCMV-specific IgG, does not correlate with the amplitude of

the memory NK cell population. Many evidence suggest a pivotal role for CD16-triggered signals in directing memory NK cell functional response (Bryceson YT et al., 2006; Fauriat C et al., 2010a). Our data, obtained on a CD16-genotyped cohort of healthy HCMV$^+$ donors, indicate that allelic variation at position 158, that affects CD16 affinity for IgG Fc region, and has been previously shown to determine NK cell effector response to antibody-coated target cells (Battella S et al., 2016; Koene HR et al., 1997; Capuano C et al., 2017), does not impact either on the establishment or on the size of the peripheral blood memory NK cell population.

Besides host factors, HCMV-dependent features may also play a role in contributing to the establishment of memory NK cell subset. A recent work by Chiara Romagnani's group reports that HCMV genetic variants for the UL40 protein correlate with a different ability to induce the memory NK cell population and functionality, highligthing the role of virus-derived factors (Hammer Q et al., 2018).

Our work has also established an *in vitro* model to explore some of the requirements for the expansion of human memory NK cells. This experimental protocol consists of a 9 days co-culture of PBMC with γ-irradiated Raji lymphoblastoid EBV$^+$ B cell line, together with IL-2.

The capability of Epstein-Barr virus-infected B cells to promote NK proliferation has been widely recognized (Granzin M et al., 2017), but no information on their effect on memory NK cell proliferation was available. Our results clearly indicate that memory NK cells are induced to strongly proliferate in these conditions, but only when RTX (anti-CD20 therapeutic mAb)-opsonized CD20$^+$ Raji cells are present. Differently, conventional NK cells comparably proliferate upon co-culture with mAb-opsonized or control Raji cells. CD16-triggered proliferation of NK cells has been previously reported, in an *in vitro* 21-day culture protocol (Lee HR et al., 2017), but the evaluation of memory NK cells was not part of this study. Our results indicate that CD16 crosslinking provides essential signals to promote memory but not conventional NK cell proliferation, thus stressing that proliferation and expansion of these two subsets may have different requirements. They also suggest the possibility that a similar mechanism may operate *in vivo*, in patients affected by B cell malignancies that undergo tumor-targeting mAb-based therapeutic schemes. Our ongoing research project is analyzing, in a prospectic

observational study, the long-term *in vivo* dynamics of memory NK cells in a cohort of Diffuse Large B Cell Lymphoma (DLBCL) patients receiving RTX-based chemoimmunotherapy.

Finally, our results indicate that CD16 affinity ligation differences that depend on allelic variants at position 158 do not affect memory NK cell *in vitro* expansion, under our experimental conditions, thus mirroring what observed *in vivo*.

It must be stressed that our *in vitro* system showed to be suitable for vigorous expansion of memory NK cells, only from individuals with a previous history of HCMV infection, independently from the size of *the in vivo* memory NK cell pool. Our data support the idea that a viral priming *in vivo* is a crucial and fundamental step in the generation of memory NK cells.

CD16 triggering is not the only stimulus in our co-culture setting. In fact, Raji cells may also provide co-stimulatory signals for NK cell proliferation and functions that are currently under investigation. In this regard, CD2 co-stimulation has been demonstrated to have a crucial role in human memory NK cell response (Liu LL et al., 2016); moreover, DNAM-1 interaction with its ligands has been shown to affect mouse memory NK cell activation (Nabekura T et al., 2014). In addition, soluble mediators and cell-cell contacts with other PBMC populations may also provide accessory signals for the proliferation of memory NK cells.

Several members of the IL-2 family have been widely shown to modulate survival, activation and proliferation of NK cells. IL-2 family cytokines share receptor components and signal transduction mechanisms, have both overlapping and different effects on NK cells, can be produced in different tissues, by different cell types and in different moments of the immune response (Parrish-Novak J et al., 2002; Pillet AH et al., 2009; Waldmann TA, 2015). In this regard, our data show that different members of the IL-2 family play distinct roles in regulating memory and conventional NK cell *in vitro* proliferation. In particular, IL-15 sustains memory NK cell proliferation in the presence of control (non-opsonized) Raji cells, while IL-2 and IL-21 do not; IL-21, at variance, induces a more potent proliferation of conventional NK cells than IL-2, while it has deleterious or no effect on memory NK cells. These results suggest that different members of the IL-2 family play non-redundant roles in the modulation of memory NK cell expansion, and that memory and conventional NK cells differently respond to the presence of these cytokines,

at least at the concentrations used in our experiments. However, it must be noted that IL-2 family cytokines may have effects on the other cell types in the PBMC suspension, as well as on Raji cells, that, despite lethal irradiation, are still viable in the initial period of the co-culture; thus, the recorded effects on memory NK cell proliferation may directly or indirectly depend on cytokine networks instaurated among the cell components of the culture. Future studies will attempt to dissect, in more controlled experimental setups, the biological requirement for memory NK cell proliferation and survival.

One of the most intriguing characteristics of memory NK cells is their enhanced Ab-dependent functional response (Lee J et al., 2015; Liu LL et al., 2016; Hwang I et al., 2012; Zhang T et al., 2013; Schlums H et al., 2015). We have confirmed that memory NK cells mediate stronger multifunctional resposes, evaluated as the percentage of cells that simultaneously produce IFN-γ and release cytotoxic granules, upon CD16 engagement with RTX-opsonized Raji cells. Moreover, our data show that such enhanced functionality is maintained after the *in vitro* culture. Interestingly, the already noted lower responsivity to stimulation with K562 prototypic "natural" target (Foley B et al., 2012b; Wu Z et al., 2013) is also conserved, upon *in vitro* proliferation.

It has been proposed that memory NK cell hyper-responsivity to CD16 aggregation could depend on the loss of FcεRIγ chain. CD16 signal transduction capability indeed relies upon coupling with CD3ζ and FcεRIγ adapter homo- or heterodimers. FcεRIγ chain has one ITAM activating domain, while CD3ζ has three of them (Lanier LL et al., 1991). It is possible that, in memory NK cells, the exclusive coupling with CD3ζ, due to the loss of FcεRIγ, results in an increased strength of CD16 downstream signals and in an amplified functional response. This would override the lower levels of CD16 on memory NK cells, as compared to conventional ones. Indeed, lower expression of CD16 is a recognized characteristic of this subsets (Hwang I et al., 2012). Here we show that this feature is conserved across CD16 genotypes, although, genetic high- and low- affinity variants of CD16A gene are expressed at different levels on the surface of NK cells, and this holds true on both memory and conventional NK cells.

Together with a lower expression of CD16 receptor, memory NK cells display a

distinct pattern of expression of other surface and intracellular molecules, such as lower expression of NKp46, another CD3ζ- and FcεRIγ-coupled activating receptor (Rollë A et al., 2014; Hwang I et al., 2012), and increased percentage of $NKG2C^+$ cells (Lopez-Vergès S et al., 2011; Muntasell A et al., 2016; Gumá M et al., 2006). The expansion of $NKG2C^+$ is thought to be driven by the interaction of NK with HCMV-infected cells. Indeed, NKG2C recognizes HLA-E loaded with viral peptides on the surface of HCMV-infected cells (Hammer Q et al., 2018). At the intracellular level, it has been shown a decreased expression of PLZF transcription factor in memory NK cells, that may also be causally linked to the loss of FcεRIγ expression (Schlums H et al., 2015). Indeed, the memory NK cell subset is characterized by a profound general transcriptional and epigenetic remodelling (Lee J et al., 2015; Luetke-Eversloh M et al., 2014; Schlums H et al., 2015). In our work we add that, although these markers are strongly modulated upon *in vitro* culture, the distinctive pattern of expression remains unaltered. These observation indicates the *in vitro* culture does not affect the peculiar memory NK cell phenotype.

We have obtained novel information on the expression of PD-1 checkpoint inhibitor and its ligand PD-L1 on memory NK cells. Such receptor-ligand axis is daily gaining interest in the scientific community for its importance, as novel biological drugs that interfere with it have been recently approved for the treatment of several cancer types, and provided great results in the fight of cancer (Alsaab HO et al., 2017).

Recently, the presence of PD-1 on a fraction of NK cells has been reported in a part of healthy individuals (Pesce S et al., 2017; Liu LL et al., 2017), while the expression of PD-L1 on NK cells is unknown.

We had the possibility to study PD-1 and PD-L1 expression on a limited cohort of donors, and could document that PD-L1 is indeed expressed on fresh human NK cells, at significantly lower levels on memory than on conventional NK cells, and that PD-L1 expression undergoes modulation upon *in vitro* culture. Our results are far from conclusive, but we believe that investigating the presence of molecules able to modulate memory NK cell functional activity and the crosstalk with other immune subsets may be a highly relevant point, in the quest for their exploitment in therapeutical settings.

In conclusion, our work contributes to the understanding of molecular factors that characterize memory NK cells and that participate to their expansion and persistence, both *in vivo* and *in vitro.*

Memory NK cells possess many interesting features that, in the future, may lead to their exploitment in therapeutical settings. Their enhanced ability to respond to CD16-stimulation may be useful in combination therapies that rely on tumor-targeting therapeutic mAb. In this context, our results can also provide useful insights for future protocols that allow their *in vitro* manipulation to the aim of adoptive transfer therapy schemes.

Moreover, their long-living ability and their enhanced capability to produce cytokines in response to CD16 engagement, may be key for the development of an anti-tumor adaptive response in therapeutic mAb-treated patients. It has been hypothesized that treatment with tumor-targeting mAb can induce a so-called vaccinal effect in patients (Abes R et al., 2010; Battella S et al., 2016; Pincetic A et al., 2014; Wittrup KD, 2017). In this mechanism, immunological death of opsonized tumor cells and subsequent uptake of tumor antigens by DC may promote DC maturation and enhance the establishment of tumor-specific adaptive responses. NK role in stimulating DC maturation and ensure the correct development of an adaptive response is more and more appreciated (Abes R et al., 2010; Martín-Fontecha A et al., 2004; Walzer T et al., 2005). Memory NK cells, with their longer lifespan and stronger responsivity to opsonized cells may amplify this process, contributing to long-term protection from relapse.

In conclusion, further studies are warranted, to clarify the biological features and to ascertain the therapeutic potentiality of this peculiar immune cell subset.

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